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Therapeutic proteins and their use in posterior eye segment diseases

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DOCTORAL THESIS
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Abstract

Since their introduction in the late 20th century, therapeutic proteins have become an irreplaceable class of pharmaceuticals and are today used to treat a wide variety of diseases ranging from arthritis, diabetes, and various cancers to more recently, for example, asthma and migraine. In ophthalmology, the treatment of certain neurodegenerative diseases of the retina, such as age-related macular degeneration and diabetic retinopathy, has been revolutionized by therapeutic proteins that combat the pathological growth of abnormal blood vessels in the retina. As retinal diseases are some of the leading causes of vision loss and blindness globally, and expected to grow in prevalence with aging populations, the importance and need for such ophthalmologic therapeutic proteins is expected to increase.

During the early development of therapeutic protein candidates, the production of functional protein in adequate amounts can often be a significant roadblock. In this thesis, the expression of soluble recombinant human ciliary neurotrophic factor (rhCNTF) – a neuroprotective protein with therapeutic potential against retinal neurodegeneration – in *Escherichia coli* was enhanced. Codon optimization of the hCNTF gene was combined with screening of different culture media, culture conditions, and fusion partners to pinpoint ideal expression conditions for rhCNTF. Following expression in the determined optimal conditions, the protein was purified with immobilized metal-ion affinity chromatography and gel filtration, and the *in vitro* activity of purified rhCNTF was demonstrated in a binding assay with its cognate receptor, CNTFRα. Overall, an 8–9 fold increase in soluble rhCNTF fraction and a 10–20 fold increase in yield was achieved, whereas earlier efforts to produce CNTF have commonly required purification from insoluble inclusion bodies and/or yielded low protein amounts. Furthermore, such a combinatorial approach is successful as a screening strategy for soluble expression and could be applied to other proteins of pharmaceutical interest.

When taken out of their natural biological milieus, most proteins are only marginally stable and susceptible to environmental perturbations. As such, the formulation of a therapeutic protein aims to protect the protein and retain its stability and biological activity, and ultimately to guarantee the therapeutic efficacy and safety throughout the lifetime of the pharmaceutical. Here, further characterization, formulation, and stability studies were carried out with purified rhCNTF. The proper folding of purified rhCNTF was observed with circular dichroism spectroscopy and the biological activity of the protein was verified in a cell proliferation study with a CNTFα expressing cell-line. After screening, two buffers were chosen as storage buffers for rhCNTF. Whereas minute changes in rhCNTF's oligomeric status were observed in only of these buffers, no changes in rhCNTF's thermal stability were observed in either buffer during the study period. As such, these results provide a basis for further formulation development for rhCNTF.

Although intravitreally injected therapeutic proteins have become the cornerstone in the management of retinal neovascularization, how and to what extent these and other proteins penetrate into the retina remains poorly understood. Here, permeation into the neural retina was observed with fluorescently labeled rhCNTF in *ex vivo* retinal explant models. Our results indicate that permeation to the CNTF-responsive target cells in the retina is not a limitation to exogenous CNTF's direct

neuroprotective actions. Moreover, our results provide further impetus to utilize *ex vivo* methods to systematically elucidate the retinal permeation and ocular pharmacokinetics of therapeutic proteins by and large.

Therapeutic proteins have not only held the helm of best-selling pharmaceuticals for some time now, but currently also represent more than 40% of new pharmaceuticals in the development pipeline. Regardless, protein drug development has been plagued with ever increasing development costs yet with fewer new drugs entering the market, and there is an urgent call to disrupt this unsustainable cycle. While reasons for failure are diverse, for therapeutic proteins the most reported are poor therapeutic efficacy and immune responses, issues which are often encountered relatively late during the drug development workflow. Therefore, it would be of utmost utility to develop methods for detecting such susceptibility before significant effort and funds are spent in the development of less than ideal candidates. In this thesis, by integrating cell-free protein synthesis in small volumes together with split-intein mediated capture and light-triggered release, a streamlined platform for rapid protein production and screening was developed. Our results provide a proof-of-principle, with successful capture and release of protein of interest, as well as protein bioconjugation achieved using hCNTF as a model protein. The developed platform can be used for the rapid screening of therapeutic protein candidate producibility, and acts as a first module to be coupled to in-line assays for monitoring e.g. the candidate's immunogenic potential, enabling such issues to be addressed/resolved already during early development stages.

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Jaakko Itkonen

Contents

List of original publications

This thesis is based on the following publications:

I **Itkonen, J.**, Urtti, A., Bird, L.E., Sarkhel, S., **2014**. Codon optimization and factorial screening for enhanced soluble expression of human ciliary neurotrophic factor in Escherichia coli. *BMC Biotechnol*. 14: 92. doi: 10.1186/s12896-014-0092-x

II **Itkonen, J.**, Tavakoli, S., Arango-Gonzalez, B., Casteleijn, M.G., Urtti, A., **2020**. Characterization and stability studies of recombinant human CNTF and its permeation into the neural retina in *ex vivo* organotypic retinal explant culture models. (Manuscript)

III Richardson, D.*, **Itkonen, J.***, Nievas, J., Urtti, A., Casteleijn, M.G., **2018**. Accelerated pharmaceutical protein development with integrated cell free expression, purification, and bioconjugation. *Sci. Rep*. 8: 11967. doi: 10.1038/s41598-018-30435-4

The publications are referred to in the text by their roman numerals (I-III). Reprinted with the permission of the publishers.

*Authors contributed equally to the work.

Additional publications that are not included in the dissertation:

1. del Amo, E.M., Rimpelä, A-K., Heikkinen, E., Kari, O.K., Ramsay, E., Lajunen, T., Schmitt, M., Pelkonen, L., Bhattacharya, M., Richardson, D., Subrizi, A., Turunen, T., Reinisalo, M., **Itkonen, J.**, Toropainen, E., Casteleijn, M., Kidron, H., Antopolsky, M., Vellonen, K-S., Ruponen, M., Urtti, A., **2017**. Pharmacokinetic aspects of retinal drug delivery. *Prog. Retin. Eye Res*. 57: 134-185. doi: 10.1016/j.preteyeres.2016.12.001

2. Serri, C., Frigione, M., Ruponen, M., Urtti, A., Borzacchiello, A., Biondi, M., **Itkonen, J.**, Mayol, L., **2019**. Electron dispersive X-ray spectroscopy and degradation properties of hyaluronic acid decorated microparticles. *Colloids Surf. B Biointerfaces* 181: 896-901. doi: 10.1016/j.colsurfb.2019.06.044

3. Kögler, M., **Itkonen, J.**, Viitala, T., Casteleijn, M.G., **2020**. Assessment of recombinant protein production in *E. coli* with Time-Gated Surface Enhanced Raman Spectroscopy (TG-SERS). *Sci. Reps*. 10: 2472. doi: 10.1038/s41598-020-59091-3

Author contribution

Publication I
The author participated in planning the protocols and experiments with supervisors. The author prepared genetic constructs, carried out protein expression studies, and the binding assay. The author participated in writing the manuscript and prepared figures.

Publication II
The author designed the experiments with supervisors and co-authors. The author performed all protein expression, purification, and characterization experiments. The author carried out culture and sample preparation for the retinal permeation studies together with co-authors and carried out data analysis. Finally, the author wrote the draft manuscript and revised it with the help of the co-authors.

Publication III
The author contributed to experimental design with supervisors and co-authors. The author prepared genetic constructs, expressed and purified protein, and obtained, analyzed and interpreted the capture and release data in the cell-free matrix and release profiles from solid surfaces. In addition, the author performed HPLC analysis of the photocleavable peptides and contributed to the writing of the manuscript and the subsequent revision based on referees' comments.

Abbreviations and symbols

Aβ	Amyloid beta
ADA	Anti-drug antibody
ADME	Absorption, distribution, metabolism, and elimination
AMD	Age-related macular degeneration
ANG	Angiopoietin
AUC	Area under the curve
BAB	Blood-aqueous barrier
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
BRB	Blood-retinal barrier
BrdU	5-bromo-2'-deoxyuridine
BSA	Bovine serum albumin
CAGR	Compound annual growth rate
CBER	Center for Biologics Evaluation and Research
CD	Circular dichroism spectroscopy
CDER	Center for Drug Evaluation and Research
CFPS	Cell-free protein synthesis
CHO	Chinese hamster ovary, a mammalian cell line
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CNTFRα	Alpha receptor of CNTF
CNV	Choroidal neovascularization
dAb	Domain antibody
DARPin	Designed ankyrin repeat protein
DDS	Drug delivery system
DLS	Dynamic light scattering
DME	Diabetic macular edema
DR	Diabetic retinopathy
EC_{50}	Concentration for half-maximal response
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency
Fab	Fragment, antigen-binding
FBS	Fetal bovine serum
Fc	Fragment, constant/crystallizable region
FcγR	Fc-gamma receptor
FcRn	Neonatal Fc receptor
FDA	US Food and Drug Administration
FITC	Fluorescein isothiocyanate
Fv	Fragment, variable
fVII	Plasma coagulation factor VII
GA	Geographic atrophy
GDNF	Glial cell line-derived neurotrophic factor
GI	Gastrointestinal
gp130	Glycoprotein 130
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HA	Hyaluronic acid
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
Hsp	Heat shock protein
IGF	Insulin-like growth factor
IgG	Immunoglobulin G

IL	Interleukin
ILM	Inner limiting membrane
IM	Intra-muscular
IMAC	Immobilized metal-ion affinity chromatography
IOP	Intraocular pressure
IV	Intravenous
IVT	Intravitreal
JAK-STAT	Janus kinases – signal transducer and activator of transcription
LC-MS/MS	Liquid chromatography–tandem mass spectrometry
LIFRβ	Leukemia inhibitory factor receptor β
mAb	Monoclonal antibody
MacTel	Macular telangiectasia
MANF	Mesencephalic astrocyte-derived neurotrophic factor
MAPK	Mitogen-activated protein kinase
M_r	Molecular mass
NAS	New active substance
NGF	Nerve growth factor
NHS	N-hydroxysuccinimide
NPDR	Non-proliferative diabetic retinopathy
PCR	Polymerase chain reaction
PD	Pharmacodynamics
PDGF	Platelet-derived growth factor
PDR	Proliferative diabetic retinopathy
PEG	Polyethylene glycol
PEDF	Pigment epithelium-derived factor
PFA	Paraformaldehyde
PLGA	poly-lactic-co-glycolic acid
PlGF	Placental growth factor
PK	Pharmacokinetics
PTM	Post-translational modification
PTS	Protein *trans* splicing
R&D	Research and development
REC	Retinal endothelial cells
RGC	Retinal ganglion cell
r_h	Hydrodynamic radius
RP	Retinitis pigmentosa
RPE	Retinal pigment epithelium
S1P	Spingosine-1-phosphate
SC	Subcutaneous
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEC	Size-exclusion chromatography
TCEP	Tris(2-carboxyethyl)phosphine
TF	Tissue factor
T_h	Temperature of hydrophobic exposure
T_m	Melting temperature
TMB	3,3',5,5'-tetramethylbenzidine
TMDD	Target-mediated drug disposition
TNF-α	Tumor necrosis factor alpha
VEGF	Vascular endothelial growth factor
VEGFR	VEGF receptor
WB	Western blot

1 Introduction

The development of new drugs is an enormous undertaking with scarce initial guarantees of success. It has been estimated that it can, on average, take over 12 years to get a new drug on the market and that up to 2.6 billion USD in costs can mount during this development and in post-approval follow-up. Regardless of the invested time and resources, most drug candidates fail before and during clinical trials due to a variety of reasons, and only a very small fraction of initial candidates are eventually launched and reach the clinic (Paul *et al*., 2010; DiMasi *et al*., 2016). Once in the clinic, it is often the profits from only the best-selling – so-called blockbuster pharmaceuticals – that eventually cover the development costs, both their own and those of all their failed, discontinued, and shelved 'peers'. It is a commonly held view that the current situation is in no way sustainable, and that a true paradigm shift is needed if new drugs are to enter the market at the current pace in the future (Scannell *et al.,* 2012).

Using proteins as pharmaceuticals to treat diseases is tempting, as they are involved in a myriad of biological processes, so much so that they are often referred to as the 'workhorses' of the body. Due to their biological functions, roles and mode(s) of action, proteins are often considerably specific in their actions and thus elicit relatively few adverse effects via off-target actions and given their 'self' origin. Moreover, their innovative and novel therapeutic applications can open possibilities to treating diseases that have been historically undruggable – hard or even impossible to treat – with traditional small-molecule drugs. This once distant idea of using proteins as therapeutics has been a tangible reality for decades now, and protein therapeutics have since become an important and continuously expanding class of pharmaceuticals, finding frequent use and an integral role in the treatment of many different diseases such as cancer, diabetes, and hemophilia (Leader *et al.,* 2008).

The mere size of therapeutic proteins alone makes them very dissimilar from small-molecule drugs (Wan, 2016). Small-molecule drugs are generally no larger than 1,000 Da in mass and are chemically comparatively simple. Therapeutic proteins, on the other hand, are macromolecules composed of amino acids as their constituent monomers, and range in mass from a few thousand Da to more than 150,000 Da; a therapeutic protein can have between a few dozen and a few hundred amino acid residues and may also include diverse post-translational modification. Furthermore, while small-molecule drugs can rather straightforwardly be described as, for example, anionic or cationic and lipophilic or hydrophilic, proteins are much more complex and hence challenging to characterize. The 20 different amino acids found in natural proteins' structures have distinctive and diverse characteristics, therefore different regions in a single protein molecule can differ significantly in aromaticity, polarity, charge and hydro/lipophilicity. Indeed, with increasing size comes increasing complexity.

Therapeutic proteins, due to their biological nature, behave differently in the body compared to small-molecule drugs (Shi, 2014). Pharmacologically – how the drug affects the body – small molecules often act as effector molecules that alter a macromolecule's function by binding to it, whereas therapeutic proteins themselves are often the affectors. Likewise, as the pharmacokinetics – how the body handles and disposes of drugs – of proteins differ from that of small-molecules', perhaps the most

tangible manifestation of all these differences is in dosing; administering therapeutic proteins in conventional dosage forms, such as capsules or tablets, is not feasible and practically all protein drugs are currently administered parenterally, most often as injections and/or infusions.

Regardless of notable advantages over small molecule drugs, proteins are pharmaceutically far from ideal and there are notable obstacles and limitations to their utilization (Morrow & Felcone 2004; Vugmeyster *et al.,* 2012; Lagassé *et al.,* 2017). As complex molecules proteins are intrinsically unstable, especially when taken out of their native biological contexts. Proteins remain stable and functional in only a narrow window of conditions; changes in the surrounding environment such as pH, ionic strength and temperature, can lead to degradation and/or denaturation, and subsequent loss of a protein's function. Whereas small-molecule drugs can be synthesized in well-defined and controlled processes, this is less feasible for most full-size proteins. Hence, they are produced by exploiting the protein synthesis 'machinery' of living organisms, i.e., an expression host. This precondition alone gives rise to significantly different requirements, for example, in their analysis, characterization, formulation, and manufacture in general. While expressed proteins might remain relatively stable when still inside the expression host, the situation often changes dramatically when the protein is extracted from the biological context of the cells and becomes exposed to widely varying conditions and environments during downstream processing. Since the manufacturing workflow can involve countless process steps, each with the potential of adversely affecting the protein, therapeutic protein production is far from trivial, but instead highly complex with oftentimes merely procuring enough pure and biologically active protein being the biggest obstacle, especially during early phases of development. Pinpointing optimal expression conditions for even a single protein can be tedious and time-consuming. Instead, using rapid screening methods to assess the overall producibility of active protein could be utilized to increase throughput and to improve the chances for success.

Ophthalmology is one such field that would benefit from improved screening methods for new therapeutic proteins candidates. Many degenerative diseases of the posterior eye segment, and more specifically the retina (the light-sensing neural tissues at the back of the eye), lead to partial or even full vision loss over time, taking a huge toll on the patients' quality of life (Awwad *et al.,* 2017). Furthermore, these diseases also exert considerable pressure on the healthcare system, especially since many of them are age-related and predicted to increase significantly in prevalence with the ageing population. Currently, the majority of both development- and clinical-stage treatments for these diseases are therapeutic proteins.

Treating diseases that affect the retina is challenging (del Amo *et al.,* 2017). Due to the physiology of the eye, this site is essentially isolated from the rest of the body and thus very difficult to reach with conventional means such as after intravenous or per oral administration. Owing to their characteristics such as large size and hydrophilicity, therapeutic proteins cannot permeate through tissues of the anterior eye segment – the outer, visible part of the eye – and cannot be administered topically (i.e., as eye drops or gels). For similar reasons, therapeutic proteins administered via the bloodstream cannot enter the retina. Hence, to achieve therapeutically efficient drug concentrations at the target site, these drugs can currently only be administered

to the posterior part of the eye as direct intravitreal (IVT) injections every 1 to 3 months. Considerable effort has been undertaken to make these drugs more amenable for administration via other routes and – even if direct injections are still unavoidable in the future – to make it possible to administer them less frequently. Nonetheless, the retinal penetration and permeation of these molecules is poorly quantitated and understood even with IVT-injected therapeutic proteins currently in routine clinical use.

This thesis focuses on therapeutic proteins, their development, and in particular their use in treating diseases of the posterior eye segment. Emphasis is placed on experimental work carried out to optimize the production of ciliary neurotrophic factor (CNTF) – a protein with potential utility in treating neurodegenerative diseases – while also addressing its characterization and the *ex vivo* retinal pharmacokinetics of the produced CNTF. Experimental results presented herein contribute to the knowledge on CNTF by 1) providing a framework for enhanced production of soluble CNTF, 2) demonstrating the applicability of using *ex vivo* methods to study retinal penetration of proteins and 3) presenting a novel platform for the rapid screening of pharmaceutically relevant proteins using CNTF as a model protein.

2 Review of the literature

2.1 The before, now, and then of therapeutic proteins

2.1.1 Dawn of the first therapeutic protein

The concept of using proteins to manage and treat diseases is not new as proteins have been applied as therapeutic agents for almost a century. Insulin was first discovered and isolated in 1921-1922 and its utilization in the treatment of diabetes mellitus was quickly established (Rosenfeld, 2002; Leader *et al*., 2008). At first, insulin purified from crude bovine and porcine pancreas extracts was used, however the supply of animal pancreata, variations in potency, and immunological reactions in some patients were among the plentiful hurdles to overcome (Leader *et al.,* 2008; Quainzon & Cheikh, 2012). Although gradual improvements in insulin purity and prolongation of the action were steadfastly achieved, insulin sourced from animals remained the only way to treat diabetes for decades (Rosenfeld, 2002; Leader *et al.,* 2008).

The pioneering work of Watson, Crick and Franklin lead to the discovery of DNA in 1953 (Watson & Crick, 1953). Work on DNA remained vigorous during the following decades, and subsequent advances in molecular biology and molecular cloning lead to development of recombinant DNA technology, which in 1978 was successfully utilized in the expression of human insulin in *Escherichia coli* bacterial cells (Goeddel *et al.,* 1979; Johnson, 1983). This work culminated in 1982 when Humulin®, the first protein obtained via recombinant DNA technology and produced in *E. coli*, received marketing approval (Johnson, 1983; Carter, 2011).

2.1.2 Maturation and sophistication

In wake of Humulin's success, other proteins followed suit and in 1986 the first therapeutic monoclonal antibody (mAb), Orthoklone OKT3® (muromonab-CD3), was approved for counteracting kidney transplant rejection (Ecker *et al.,* 2015), followed by with Roferon A® (Interferon α) for the treatment of, i.a., hairy cell leukemia (Gutterman, 1994). Notable following breakthroughs include the step-wise humanization of mAbs: the first chimeric antibody fragment ReoPro® (abciximab), was approved in 1994 while the year 1997 saw the approval of rituximab (Rituxan®), the first chimeric full-size mAb (Brekke & Sandlie, 2003) – and also the first mAb implemented in oncology (Pierpont *et al.,* 2018) – as well as the first humanized mAb Zenapax® (daclizumab) (Almagro & Fransson, 2008). Ultimately, the first fully human mAb Humira® (adalimumab), and the first to be derived via phage display technology, was authorized in 2002 (Osbourn *et al.,* 2005). Other technological milestones include the launch of the first Fc-fusion protein therapeutic Enbrel® (etanercept) in 1998 (Watier & Reichert, 2017), the 2006 approval of Atryn® (antithrombin alfa) as the first approved biological[1] obtained from a transgenic animal (Carroll, 2006), the 2012 the approval of the first plant-based biopharmaceutical,

[1] The terms with products of pharmaceutical biotechnology are often used interchangeably and their meanings can differ between fields of science, inside the industry and between regulatory bodies. While the terms 'biological', 'biological medicine' and 'biological product' are used to refer to, e.g., therapeutic proteins, vaccines, nucleic acid drugs, gene therapy, and cell therapy, 'biological' will herein refer solely to protein-based therapeutics, unless otherwise specified.

Elelyso® (taliglucerase alfa), (Fox, 2012) and of Poteligeo® (mogamulizumab), the first glyco-engineered mAb (Beck & Reichert, 2012), and ultimately the emergence of cell-free protein synthesis (CFPS) for the production of protein therapeutics (Casteleijn *et al.,* 2013),

2.1.3 Indications and classification of therapeutic proteins

Biologicals are used for varying indications (Walsh, 2014). Whereas hematology and oncology are the biggest therapeutic areas for biologicals, they have been clinically applied i.a. in dermatology, endocrinology, immunology and rheumatology (Lagassé *et al.,* 2017). Although the majority of both existing and emerging biologicals still fall within these 'traditional' therapeutic areas (Walsh, 2014), the use of biologicals has in recent years also branched into treatment of asthma (Markham, 2018), chronic pain (Patel *et al.,* 2018), hypercholesterolemia (Chaudhary *et al.,* 2017), infectious diseases (Sparrow *et al.,* 2017), multiple sclerosis (Heliopoulos & Patousi, 2018), and even migraine prevention (Tepper, 2018). The use of proteins, however, is not solely limited to therapy as they are also utilized in diagnostics and as vaccines (Leader, 2008; Walsh, 2014).

Regardless of various applications, therapeutic proteins can be categorized in several ways, e.g., based on their molecular types and on molecular mechanisms of their activities (Carter, 2011; Dimitrov, 2012; Usmani *et al.,* 2017). A now commonly accepted classification system organizes protein therapeutics based on their function and therapeutic applications (Leader *et al.,* 2008; **Fig. 1**). Accordingly, protein therapeutics are organized depending on their pharmacological activities into groups where proteins: I) have enzymatic or regulatory activity, II) have special targeting activity, III) are used as vaccines, and IV) are used in diagnostics. Groups I & II can be further subdivided based on more specific physiological/therapeutic roles of the proteins as they: Ia) replace a deficient or abnormal protein, Ib) augment an existing pathway, Ic) provide a novel function or activity, IIa) interfere with a molecule or organism, and IIb) deliver and/or target a cargo. Groups III and IV are subdivided based on how the proteins are used as: vaccines against IIIa) deleterious foreign agents, IIIb) autoimmune diseases, IIIc) cancer, and as IVa) *in vivo*, or IVb) *in vitro* diagnostics.

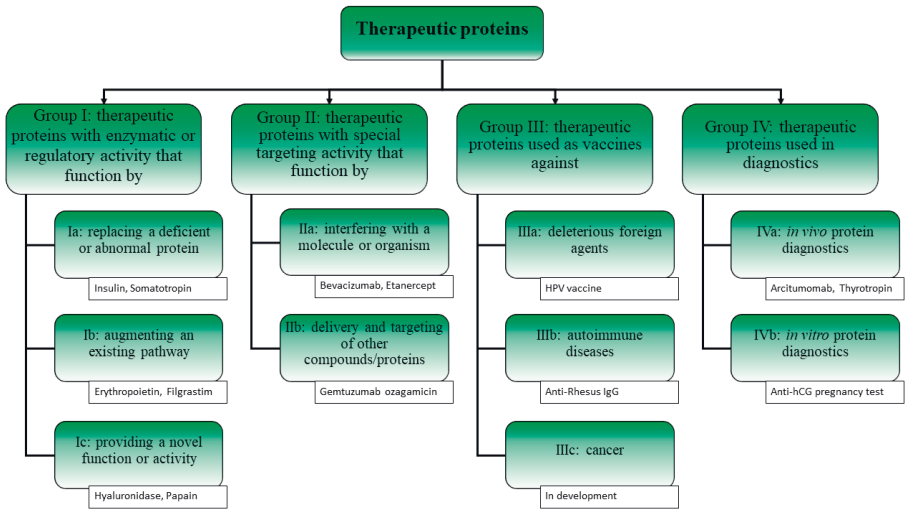

Figure 1. *Classification of therapeutic proteins (adapted and modified from Leader et al., 2008). Examples of each subgroup have been given in boxes below the group definitions.*

2.1.4 The global therapeutic protein market

Aside from some ebb and flow in annual approval numbers, the rate of new biologicals appearing on the market has not shown signs of slowing. On the contrary, the share of biologicals among new active substances (NAS) to receive marketing approval from the US Food and Drug Administration (FDA) and European Medicines Agency (EMA) has been approximately 30% in recent years (EMA, 2016–2020; FDA, 2009, 2010, 2012–2020; Mullard, 2018, 2019; **Fig. 2**).

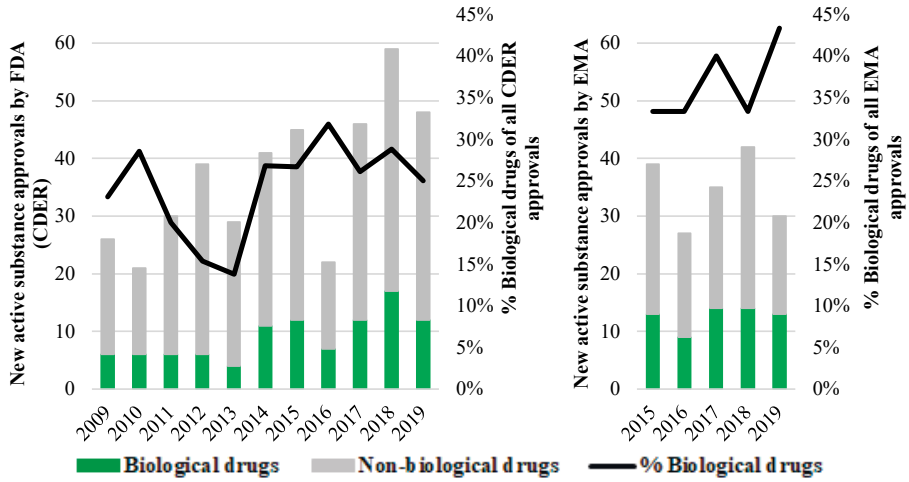

Figure 2. *Approvals of new active substances. a) FDA[2] 2009-2020; data are from Drugs@FDA b) EMA 2015-2018; data are from EMA, 2016–2020.*

[2] Review of biologicals by FDA is divided between Center for Drug Evaluation and Research (CDER) and Center for Biologics Evaluation and Research (CBER) depending on the therapeutic class. For consistency, only CDER-regulated biopharmaceuticals, i.e., mAbs and therapeutic proteins, are included here.

The evidence indicates that these numbers are unlikely to go down; in 2013 over 400 drugs in clinical trials or under FDA's review were biologicals (PhRMA, 2013) and, in 2019, nearly 40% out of all NASs in the research and development (R&D) pipeline were biologicals (Pharmaprojects, 2019; **Fig. 3**). Although emerging (advanced) therapies, e.g., antisense, cell therapy and gene therapy, are in included in these numbers, mAbs and other proteins remain the most significant biopharmaceuticals (PhRMA, 2013; Lagassé *et al.,* 2017; Morrison, 2018; Walsh, 2018; **Table 1**). Moreover, the expansion rate of new mAbs in the R&D pipeline has, in recent years, consistently surpassed that of small molecules and the overall average of the top-25 followed drug categories (Pharmaprojects, 2015–2019; **Fig. 4**).

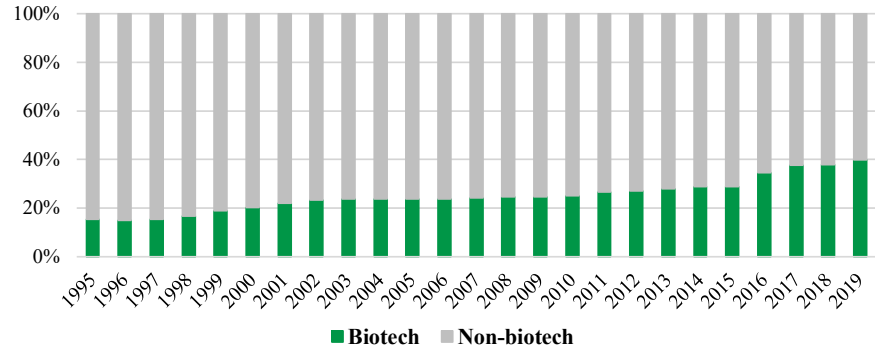

Figure 3. *Share of biopharmaceuticals in the Pharma R&D pipeline 1995-2018. Data from Pharmaprojects, 2019.*

Table 1. *Share of pipeline drugs by origin.*

	Year	2014	2015	2016	2017	2018	2019
Origin of pipeline drug (%)	Chemical, synthetic	56.4	56.0	55.8	53.7	52.2	51.9
	Biological, antibody	10.5	10.5	10.9	11.5	11.8	12.8
	Biological, recombinant protein	7.3	7.3	6.6	5.9	5.4	5.3
	Biological, protein	3.3	3.4	3.3	3.7	3.3	3.3
	Chemical, synthetic peptide	3.1	3.3	3.2	3.2	2.9	2.7

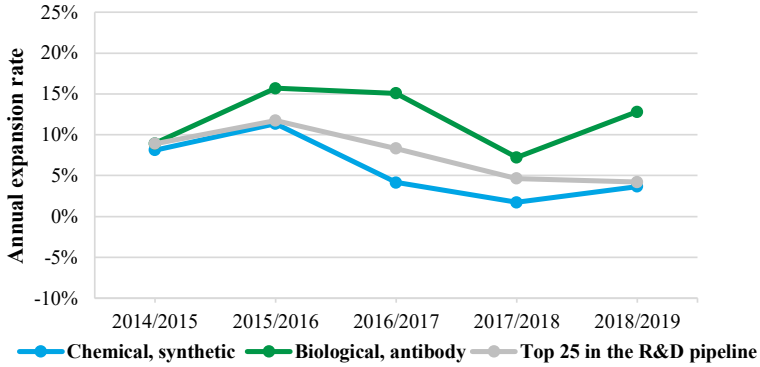

Figure 4. *Annual expansion rates of chosen chemical classes in the R&D pipeline 2014-2018. Data are from Pharmaprojects 2015–2019.*

In both 2017 and 2018, 7 out of the top 10 pharmaceuticals by global sales were biologicals, out of which 6 in 2017 and all 7 in 2018 were mAbs (Urquhart, 2018;

Urquhart, 2019). Furthermore, in 2024, 31 out of 50 globally top selling pharmaceuticals are expected to be biologicals (EvaluatePharma, 2018). Likewise, revenues are expected to remain high and in 2018, 10 out of 20 most valuable R&D projects were biologicals. Projections for the global therapeutic proteins market predict a compound annual growth rate (CAGR) of 10.6% and 9.4% p.a. up to 2019 (BCC Research, 2015) and 2024 (Research and Markets, 2018), respectively. Furthermore, biotechnology products are predicted to reach a 31% market share and to represent 52% of the Top 100 drug sales by 2024, up from 25% and 49% in 2017, respectively, underlining the industry's trust and commitment with biologicals as NASs (EvaluatePharma, 2018).

Studies comparing the success rates and approval times of small molecules and biologicals in clinical trials have revealed that biologicals tend to fare better (Reichert, 2003; Kola & Landis, 2004; DiMasi *et al.,* 2010; Hay *et al.,* 2014; Hwang *et al.,* 2016; Smietana *et al.* 2016). However, despite intensive research, successful approvals, and thus somewhat warranted high expectations, biologicals have not been able to break the so-called Eroom's law, the observation that in spite of technological advancements, drug discovery and development is getting increasingly more expensive yet yielding fewer new drugs (Scannell *et al.,* 2012). While more resources are funneled to develop new drugs, nonetheless a smaller fraction of drugs succeed in clinical trials and eventually gain market access (Hay *et al.,* 2014; Schuhmacher *et al.,* 2016). Despite the emergence and success of biologicals, R&D efficiency for biologicals is not markedly better (DiMasi *et al.,* 2010) and the unsustainable trend remains undisrupted, calling for novel and innovative approaches to address the issue (Schuhmacher *et al.,* 2016). Limitations and obstacles, such as long production times and high costs, can hamper and even prevent more widespread use of biological drugs; a prime example of emerging innovations and solutions to counteract this is the Open Insulin Project, an initiative for circumventing regulations and developing insulin production in a 'do-it-yourself' manner on a city, community, or even individual scale to make this increasingly costly drug more accessible (Gallegos *et al.,* 2018).

2.2 Life of trouble with therapeutic proteins

2.2.1 Protein structure and folding

Therapeutic proteins are proteins that are applied medically to diagnose, treat, prevent, and cure diseases. Proteins consist of up to 20 different proteinogenic L-amino acids linked together by peptide bonds (Nelson & Cox, 2008). A polypeptide chain is called the primary structure and can range significantly in length and amino acid content between different proteins (**Fig. 5a**). Therapeutic peptides are polypeptide chains and hence chemically related molecules, but as they generally have less than 50 amino acids and a molecular mass below 10 kDa, they are distinct in many aspects such as pharmacokinetics (Diao & Meibohm, 2013), and for these reasons are not discussed further in this thesis.

The high complexity and functions of proteins do not solely stem from the simple polypeptide chains, but the higher-order conformations they fold into (Kessel & Ben-Tal, 2018). Interactions, such as hydrogen bonds, ionic bonds, and hydrophobic interactions between amino acids allow regions of the polypeptide chain

to fold into simple energetically favorable ordered conformations known as secondary structures, such as α-helices (**Fig. 5b**), β-sheets (**Fig. 5c**), and turns. Compacting of the secondary structures into motifs held together by aforementioned interactions, and further stabilized e.g. by interchain disulphide bridges, result in the tertiary structure (**Fig. 5d**). This native structure and its features give rise to functions and biological activities of proteins. Some proteins have a multimeric structure where individual tertiary structures oligomerize to quaternary structures (**Fig. 5e**). Moreover, many proteins also have one or more glycan moieties – polysaccharides comprised of various sugar molecules – attached to specific amino acids as integral parts of their structure in addition to other post-translational modifications (PTMs), adding yet another layer of structural complexity and heterogeneity (Goh & Ng, 2018).

Despite several types of contributing interactions, the major overall driving force in protein folding and stabilization of the structure is considered to be the so-called hydrophobic effect (Tanford, 1997; Chandler, 2005; Nelson & Cox, 2008; Compiani & Capriotti, 2013); the water molecules' strong propensity to form and maintain hydrogen bonds leads to the burial of most non-polar, hydrophobic residues into the protein core – away from aqueous solvent – whereas polar residues remain exposed to solvent at the protein surface.

Proteins' attributes, e.g. molecular mass, hydrodynamic size, charge distribution, hydro- and lipophilicity, and other physico-chemical properties, play a role in their functions in the body and give rise to complex and heterogenous structures (Nelson & Cox, 2008; Kessel & Ben-Tal, 2018a) and their interactions in various complexes (Kessel & Ben-Tal, 2018b). It is not difficult to fathom why therapeutic proteins differ so much and in so many aspects from small-molecule drugs; a side-by-side comparison already reveals the most obvious differences: size and structural complexity (**Fig. 5f**). The size difference between acetylsalicylic acid (Aspirin®), a small-molecule drug of 180 Da with a radius of approximately 0.5 nm, and a monoclonal antibody of 150,000 Da with a radius of 5.5-6.5 nm translates roughly to that between a green pea and a grapefruit. Other differences are discussed in following chapters, and a summary of certain key differences is compiled in **Table 2**.

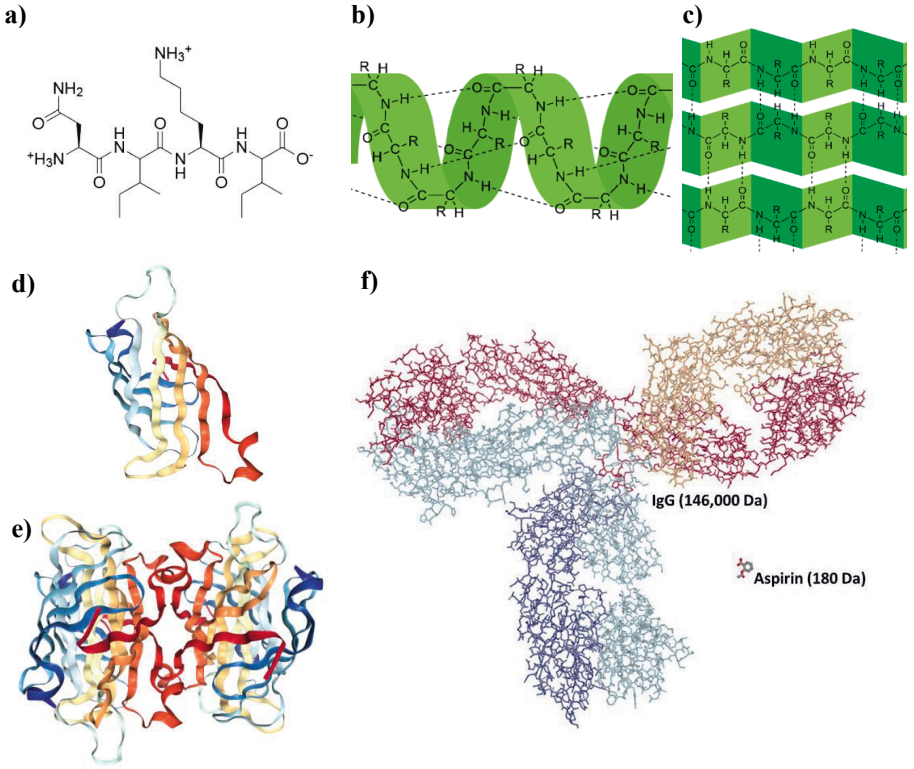

Figure 5. ***Structural features of proteins. a)*** *Primary structure of tetrapeptide asparaginylisoleucyllysylisoleucine,* ***b)*** *α-helix,* ***c)*** *(anti-parallel) β-sheet,* ***d)*** *tertiary structure (a β-barrel monomer), and* ***e)*** *quaternary structure (tetramer of four β-barrels) of streptavidin.* ***f)*** *Size and structural complexity comparison between pembrolizumab (Mr=146,000 Da), an IgG protein therapeutic, and Aspirin (Mr=180 Da), a small-molecule drug. Images for Streptavidin (4GJS), Pembrolizumab (5DK3), and Aspirin® have been procured from The Research Collaborative for Structural Bioinformatic (RCSB) Protein Data Bank (www.rcsb.org).*

Table 2. *Comparison of small-molecule drugs vs. biologicals (adapted and modified from Morrow & Felcone, 2004; Mahmood & Green, 2007; Schellekens, 2009; Shi, 2014; Andrews et al., 2015; Ferl et al., 2016; Oo & Kalbag, 2016; Wan, 2016).*

	Property		Small-molecule drugs	Biologicals (proteins)
Chemistry	**Molecular weight (M_r)**		< 1000 Da	Up to 170,000 Da
	Drug substance		Single entity; chemically pure	Generally uniform; related molecules/ fragments present
	Structure		Simple, well-defined	Complex (e.g., tertiary structure, PTMs), heterogenous, defined by manufacturing process
	Physicochemical properties		Well-defined	Complex, depends on the environment
	Physicochemical characterization		Well-defined	Full characterization is complex if not impossible
	Stability		Stable	Metastable, susceptible to external conditions, e.g., during manufacturing
Pharmacokinetics	**ADME & tools**		Extensive ADME understanding, tools available	Understanding and tools of ADME still evolving
	Administration route		All routes, per oral usually possible	Parenterally as injections/infusions (IV, SC, IM, IVT); per oral not feasible
	Absorption		Rapid entry into systemic circulation via blood capillaries	If not administered systemically, initial distribution via lymphatic system upon e.g. SC administration
	Distribution volume (V_d)		Sometimes high V_d, variable, protein binding and transporters play a role, distribution to many organs/tissues/cells	Lower V_d, distribution often limited to plasma and/or extracellular fluids due to membrane impermeability
	Clearance mechanisms	**Metabolism**	CYP P450 enzymes & phase II enzymes; active and non-active metabolites	Proteases and peptidases ubiquitous in the body; catabolism to non-active peptides and single amino acids that are used *de novo* in protein synthesis
		Renal	Renal excretion	Renal excretion possible for small proteins and fragments; enzymatic degradation
		Hepatic	Metabolism, biliary excretion	Non-specific proteases and peptidases
		Target-mediated	Rare	Yes
		Intestinal	CYP P450 enzymes, transporters	Not relevant due to parenteral administration
	Half-life ($t_{1/2}$)		Short; several hours to 24 h	Long; days to weeks
	Clearance		Mostly linear, can be saturable	Slow clearance
	Drug-drug interactions		Common, both PK and/or PD related	Less common; PD related
	PK Analysis		Drug molecule and its metabolites	Drug molecule and anti-drug antibodies

Table 2 continued.

	Property	Small-molecule drugs	Biologicals (proteins)
Pharmacology	**Potency and selectivity**	Selective and non-selective	Highly potent and highly selective
	Target	Varies, intracellular and CNS possible	Membrane impermeable; targets extracellular and outside CNS
	Toxicity	On- and off-target toxicity	Low toxicity; mainly exaggerated pharmacology
	Immunogenicity	Rarely observed	Frequently observed; often of clinical significance
	PK/PD	Due to dominance of non-target binding, PK usually not driven by PD	PK and PD mechanistically connected; target-mediated drug disposition
R&D / Preparation	**Quantification**	LC-MS/MS methods	Mostly ligand binding assays (ELISA); recently LC-MS/MS
	Production	Chemical synthesis, predictable and well defined, standardized and reproducible	Manufactured in living cell cultures, up to 5000 critical process steps, batch-to-batch variation, engineering possible
	Formulation	Complex, diverse	Simple aqueous or freeze-dried formulations
	Species reactivity	Multiple animal models relevant	Many animal models irrelevant

ADME, absorption, distribution, metabolism and elimination; CNS, central nervous system; ELISA, enzyme-linked immunosorbent assay; IM, intramuscular; IV, intravenous; IVT, intravitreal; LC-MS/MS, liquid chromatography – tandem mass spectrometry; PD, pharmacodynamics; PK, pharmacokinetics; PTM, post-translational modification; SC, subcutaneous.

2.2.2 Stability of therapeutic proteins

As large, complex, and flexible molecules, proteins are fragile and susceptible to external factors and stimuli (Morrow & Felcone, 2004). Compared to the unfolded state, the folded (native) state of proteins is energetically only marginally more favorable, and proteins remain chemically and physically stable only within narrow, protein-specific conditions and environments favoring this native state (Rathore & Rajan, 2008; Andrews *et al.,* 2015). As the functionality, and therefore pharmaceutical activity, of therapeutic proteins is dependent on the intricate protein structure and the maintenance of this delicate three-dimensional conformation, therapeutic protein stability is of utmost importance (Radhakrishnan *et al.,* 2017).

The protein concentrations and milieus in which therapeutic proteins are handled and formulated in can be far removed from each protein's 'normal', physiological conditions (Lipiäinen *et al.,* 2015). Differences from the protein-specific optimal pH, temperature, and ionic strength as well as perturbations in these factors, along with stress from shear and contacts with interfaces, surfaces, and impurities during processing can all adversely affect therapeutic proteins (Frokjaer & Otzen, 2005; Patel *et al.,* 2011; Gervasi *et al.,* 2018). These different factors may interfere with a therapeutic protein's chemical composition and/or its physical state (Manning *et al.,* 2010). For example, destruction and formation of chemical bonds can destabilize the protein structure and/or trigger proteins to unfold. Furthermore, as its biotransformation can impact efficacy, maintaining stability in the *in vivo* milieu after administration is of utmost importance (Loyet *et al.,* 2019).

The numerous protein degradation pathways further complicate the picture; degradation may initiate via one pathway and proceed through other and even multiple pathways (Rathore & Rajan, 2008). Regardless of the degradation mechanisms and pathways, therapeutic protein instability can result in the alteration or even abolishment of the proteins' functions, most commonly manifesting as loss of therapeutic efficacy and enhanced immunogenicity (Patel *et al.,* 2011; Lipiäinen *et al.,* 2015; Nejadnik *et al.,* 2018). Whereas protein degradation may be less detrimental and reversible in the short-term, irreversible degradation and irretrievable loss of protein structure often takes place, especially in the long-term.

2.2.3 Production of therapeutic proteins

Therapeutic protein engineering

(Native) proteins fall short in many desirable and required features for optimal druggability, such as solubility, immunogenicity, biological half-life, etc. (Szymkowski, 2005; Dingermann, 2008). Therapeutic protein engineering is now commonly carried out to augment, incorporate and modify such pharmaceutical attributes and qualities. The currently available approaches and techniques are diverse, with certain strategies having gained a firm foothold.

Fusion proteins are often constructed to extend the biological half-life, whereas a protein's glycosylation pattern may be altered to influence receptor-binding (Lagassé *et al.,* 2017) or, in the case of the glycoengineered erythropoietin darbepoetin-α, to increase the biological half-life (Dingermann, 2008). Similarly, PEGylation imparts protection against enzymatic degradation, diminishes immunogenicity and renal clearance by increasing a therapeutic protein's size, which translates into extended circulatory half-life and improved protein stability (Szymkowski, 2005; Shi, 2014; Lagassé *et al.,* 2017). Aside from such 'extrinsic' modifications, a protein itself and, for example, it's primary sequence can be engineered to influence pharmacokinetics, ligand/receptor binding, stability, etc. Dramatic improvements in protein stability have been achieved with minimal amino acid changes in certain cytokines (Szymkowski, 2005), while pegvisomant is a human growth hormone variant engineered to compete with the endogenous protein, i.e., to act as a receptor antagonist (Dingermann, 2008).

Upstream processing – therapeutic protein expression

Therapeutic proteins are generally not amenable for production by chemical synthesis because of their large size and complexity, but instead need to be expressed as recombinant proteins in living cells with bacterial, mammalian, and yeast systems being the most commonly utilized (Lagassé *et al.,* 2017; Ryman & Meibohm, 2017; Wells & Robinson, 2017). The required expression system, culture conditions, etc. are mostly dictated by the protein and its (possible) PTMs, such as glycosylation. Reflecting the high number of mAbs, approximately 70% of recombinant therapeutic proteins on the market are produced in glycosylation-performing mammalian-based systems, such as Chinese hamster ovary (CHO) cells (Stech & Kubick, 2015), whereas with recent approvals this relative share has now gotten closer to 80% (Walsh, 2018).

In recent years, there has been increased interest in cell-free protein synthesis (CFPS) based platforms for therapeutic protein production (Zawada *et al.,* 2011; Lee & Kim, 2018; Khambhati *et al.,* 2019). These *in vitro* systems can be derived from both prokaryotic and eukaryotic cells and offer notable advantages in contrast to traditional, living cell-based protein expression systems. For example, the cytotoxicity of expressed protein is not a limiting factor as cell maintenance is not needed, allowing for the expression of toxic proteins and other 'difficult-to-express' proteins. The system's openness – the cell lysis has taken place before protein production – allows the protein synthesis conditions to be more tightly controlled, and the capacity and resources to be focused toward target protein production. The open nature of these systems also enables the incorporation of unnatural amino acids, which can in turn be utilized, e.g., in protein engineering and bioconjugation (Casteleijn *et al.,* 2013; Stech *et al.,* 2014; Khambhati *et al.,* 2019). Moreover, CFPS systems can utilize linear DNA templates, allowing for streamlined parallel expression of various proteins, effectively shortening the production times from weeks and months to mere days (Stech & Kubick, 2015; Lee & Kim, 2018). Different CFPS systems have so far been utilized in the small-scale production of many therapeutic proteins, such as erythropoietin (Sullivan *et al.,* 2016), antibody fragments (Stech *et al.,* 2014) and full antibodies (Buntru *et al.,* 2015; Stech *et al.,* 2017).

Although the industrial-scale production of biologicals using CFPS technologies is still in its infancy, Sutro Biopharma, for example, has utilized their *E. coli*-based platform to produce an active cytokine at a 100-liter scale with yields comparable to more established, living cell-based platforms (Zawada *et al*., 2011). The potential of these technologies is substantial, and they can be envisioned to be applied in the point-of-care production of niche biologicals – therapeutics for orphan diseases or patient-specific treatments – that do not require large batch sizes (Sullivan *et al*., 2016; Timm *et al*., 2016; Ogonah *et al*., 2017). Broader possibilities can be expected to arise with technological advancements, as for example tight control of target protein glycosylation has recently been achieved in *E. coli* based CFPS for the first time (Jaroentomeechai *et al*., 2018).

Downstream processing – therapeutic protein processing and formulation

Regardless of utilized expression systems, after production, proteins are commonly subjected to several steps of protein purification and evaluation before a therapeutic protein is finally formulated into a finished drug product (Lagassé *et al.,* 2017; Wells & Robinson, 2017). The instability of proteins makes their pharmaceutical development and manufacture complex and often challenging. Proteins may be susceptible to changes in the manufacturing steps and the involved processes must be meticulously optimized and strictly controlled to minimize batch-to-batch variation in the finished drug products (Crommelin *et al.,* 2005; Rathore & Rajan, 2008; Schellekens, 2009; Ryman & Meibohm 2017). Due to the manufacturing stages influencing the end-product and its quality, insofar that in therapeutic protein manufacturing the process itself is considered to 'be' the product (Baumann, 2006). Moreover, further complicating their manufacture is the fact that proteins are highly heterogenous and as each protein can be considered somewhat unique, production challenges and manufacturing requirements are solved on a case-by-case basis,

necessitating unique approaches and solutions (Andrews *et al.,* 2015; Wells & Robinson, 2017). Large size together with structural complexity and heterogeneity tends to translate into more challenges. Factors other than the protein itself can also dictate the formulation requirements. For example, with IVT injections high doses are often needed for therapeutic efficacy and as the FDA limits the maximal injectable volume, therapeutic proteins for IVT administration need to be formulated at very high concentrations, which are in turn, often highly viscous and thus poorly syringeable (Mandal *et al.,* 2018). Likewise, the excipients approved for intravitreal injections are very limited, thereby constraining stabilization possibilities (Wakshull *et al.,* 2017).

As already discussed, therapeutic proteins are susceptible to many destabilizing external stimuli and they can degrade through numerous different pathways (Manning *et al.,* 1989; Patel *et al.,* 2011). Hence, proteins require protection through stabilization – especially in the case of unmodified native proteins – to ensure therapeutic efficacy and safety, and to retain product integrity (Szymkowski, 2005; Radhakrishnan *et al.,* 2017). As different process-stresses can potentially impact the efficacy and activity of therapeutic proteins, careful control of process parameters is required to alleviate these concerns (Rathore & Rajan, 2008).

Therapeutic protein formulation aims to identify a final pharmaceutical composition in which the protein remains stable and resistant towards the stresses during manufacturing, storage, and administration (Frokjaer & Otzen, 2005; Gervasi *et al.,* 2018). Common therapeutic protein formulation and stabilization strategies include the optimization of buffer, pH, and osmolarity as well the inclusion of stabilizing excipients, such as chelating agents, detergents, polymers, surfactants, sugars and polyols, as well as free amino acids (Frokjaer & Otzen, 2005; Szymkowski, 2005; Jorgensen *et al.,* 2009; Manning *et al.,* 2010; Gervasi *et al.,* 2018). As mentioned, therapeutic protein engineering can be carried out to enhance stability; PEGylation, i.e., conjugating polyethylene glycol (PEG), and glycoengineering can improve protein stability whereas modifying the protein itself via, e.g., site-directed mutagenesis, has been utilized to improve both the chemical and physical stability of therapeutic proteins. After release, product (mis)handling – either in a hospital setting or by the patient – can still expose therapeutic proteins to various stresses that may compromise therapeutic protein stability, and the safety and efficacy thereafter (Nejadnik *et al.,* 2018). Clearly, maintaining stability is of paramount importance in all stages of a biological's lifetime.

However, the different mechanisms of stabilization are not comprehensively resolved and may in fact differ between individual therapeutic proteins. Hence, although certain strategies and technologies have become somewhat standard, it needs to be noted that currently there is no catch-all approach capable of universally resolving therapeutic protein stability challenges, and that formulation development must be based on understanding each individual protein's unique requirements and mechanisms of stabilization (Jorgensen *et al.,* 2009; Lipiäinen *et al.,* 2015; Wang, 2015).

2.2.4 Systemic pharmacokinetics and administration of therapeutic proteins

Since the subject of therapeutic protein pharmacokinetics (PK) is broad, the following is a very limited summary in which some omissions and overt generalizations are unavoidable. Key aspects are also presented in **Table 2**.

Therapeutic proteins' PK differs considerably from that of small-molecule drugs' in most aspects of drug disposition, i.e., absorption, distribution, metabolism and elimination (ADME), as the involved mechanisms and pathways differ (Lin, 2009). Most of these differences can be attributed to protein properties, perhaps most importantly their high molecular weight and size, and susceptibility to degradation owing to their biological origin (Mitragotri *et al.,* 2014). Due to the former, therapeutic proteins do not readily permeate through biological membranes, such as cell membranes and mucous membranes, a feature in and of itself a significant determinant of their PK.

Absorption and distribution

Intestinal digestive enzymes break protein consumed in food down into smaller peptides and amino acids before their absorption from the gut lumen to the circulation. *Per os* administered therapeutic proteins are not an exception: they are unstable in this hostile proteolytic environment and cannot penetrate the epithelium of the gut, resulting in poor bioavailability and practically nullifying the use the oral administration route. However, given the associated advantages such as non-invasiveness, ease of dosing and high patient compliance, its realization is nonetheless considered the 'holy grail' of therapeutic protein dosing and different means – ranging from the engineering, conjugation, and chemical modification of therapeutic proteins to their encapsulation in micro- and nanoparticle drug delivery systems – to achieve this are continually explored (Shen, 2003; Patel *et al.,* 2014; Shah, 2015).

Currently, to attain adequate systemic exposure therapeutic proteins are administered parenterally as injections or infusions via intravenous (IV), intramuscular (IM) and subcutaneous (SC) routes, or for local effect, e.g., as IVT injections (Vugmeyster *et al.,* 2012; Shah, 2015). Although direct systemic delivery, avoiding pre-systemic degradation, is possible with IV administration, this may not provide the ideal concentration-time profile and is impractical (Tang *et al.,* 2004). While the SC route is suitable for self-administration and allows larger injection volumes, the bioavailability upon IM and SC administration may be limited, for example, by local blood flow and on-site enzymatic degradation (Wang *et al.,* 2008; Vugmeyster *et al.,* 2012; Shah, 2015). Both IM and SC administered biologicals absorb slowly, entering the circulation via convection through lymphatic vessels and by diffusing into capillaries at and near the injection site, the former being more dominant with high M_r biologicals (Tang *et al.,* 2004).

Absorption via other parenteral routes is also possible and many biologicals have been shown to successfully enter circulation upon, for example, nasal and pulmonary administrations (Patel *et al.,* 2014). Nasal administration of biologicals is of interest as by circumventing the blood-brain barrier (BBB) it may, for example, allow for the successful central nervous system (CNS) delivery of neurotrophic factors to treat neurodegenerative diseases (Thorne & Frey, 2001; Yi *et al.,* 2014). Whereas there are justified concerns of achieving clinically efficacious levels in the CNS with

this administration route (Thorne & Frey, 2001; Vaka *et al.,* 2009), intranasally administered biologicals have nonetheless been documented to reach higher CNS concentrations than upon IV administration, and to elicit pharmacological actions (Thorne & Frey, 2001; Hong *et al.,* 2019). While pulmonary delivery offers convenient delivery to a site with a large, highly vascularized surface area available for absorption, there are plenty of obstacles such as local enzymatic activity in the alveoles, potential for local effects of inhaled therapeutics/excipients, and considerable formulation challenges that complicate the development of inhalable biologicals (Tang *et al.,* 2004). This has nonetheless been achieved with inhalable formulations for the treatment of cystic fibrosis and diabetes, and many therapeutic protein products for pulmonary delivery are currently in development (Morales *et al.,* 2017).

Owing to poor membrane permeability, the tissue distribution of biologicals is limited and generally restricted to the plasma and the interstitial compartment. In addition to protein properties such as molecular size, charge, target and off-target binding, the administration route as well as tissue physiology also contribute to protein biodistribution (Vugmeyster *et al.,* 2010; Shah, 2015). Aside from therapeutic protein size and charge, extravasation to tissues also depends on vascular porosity and takes place mostly via convection and to a lesser extent via diffusion. Other mechanisms such as FcRn-mediated transcytosis, active tissue uptake, and binding to carrier and transport proteins can influence distribution of certain biologicals (Tang *et al.,* 2004; Baumann, 2006; Lin, 2009). However, poor tissue distribution does not necessarily paint the full picture; as e.g. mAbs that have their putative targets in tissues can, regardless of limited tissue distribution, show that the limited tissue penetration can be sufficient for therapeutic efficacy (Vugmeyster *et al.,* 2012).

Biologicals have so far been predominantly used against extracellular or cell-surface targets, and excluded from intracellular targets (Mitragotri *et al.,* 2014). Although therapeutic protein entry to cells is hampered by poor permeation through cell membranes, an even more formidable obstacle is that the internalized proteins are often not free in the cytosol. Instead they are trapped in vesicular structures and commonly destined for degradation processes, effectively preventing them from reaching their cytosolic targets (Fu *et al.,* 2014). On a similar note, the physiology of the intact BBB effectively obstructs the penetration of biologicals to the CNS (Thorne & Frey 2001). Accordingly, successful CNS and intracellular targeting of therapeutic proteins will require creative and sophisticated solutions and while varied approaches have been explored – some even in clinical trials – to date none have yet entered the clinic (Dinca *et al.,* 2016; Fu *et al.,* 2014; Yi *et al.,* 2014; Guillard *et al.,* 2015).

Metabolism and elimination

Therapeutic proteins are broken down via the same pathways as endogenous proteins, i.e., enzymatically by proteases and peptidases in various organs and tissues. Depending on a therapeutic proteins' properties, this proteolysis can take place in locations including the liver, kidneys, blood, as well as locally at administration sites (Tang et al., 2004). Therapeutic protein properties and their physiological roles are mirrored by their clearance; protein hormones are naturally short-lived as this allows for tight regulation of their action whereas, for example, antibodies and albumin have

a significantly longer sojourn in the body since their action over longer periods of time is warranted. Hence, the pathways of metabolism and elimination of biologicals can largely be protein dependent.

Although the renal excretion elimination route is considered an important elimination pathway for many small-molecule drugs and their (active) metabolites, when considering therapeutic proteins and their degradation products, most often the excretion of unchanged protein into the urine is negligible (Baumann, 2006, Vugmeyster *et al.,* 2012). Glomerular filtration is selective on protein size, structure and charge. While proteins and degradation products smaller than the cut-off value for retinal filtration (60 kDa), and in particular biologicals smaller than 30 kDa can be eliminated by excretion into the renal tubules, the major degradation pathway is nonetheless via enzymatic proteolysis in the nephrons after glomerular filtration (Lin, 2009; Vugmeyster *et al.,* 2012).

Clearance can also take place via the liver. Biologicals can be taken up into hepatic cells, e.g., via pinocytosis and receptor-mediated transport processes and subjected to intracellular enzymatic elimination (Baumann, 2006). For example, certain glycoprotein receptors, such as lectins expressed on hepatocytes and hepatic endothelial cells are responsible for recognition and internalization of glycosylated proteins and may therefore significantly contribute to their clearance (Lin, 2009). Proteins can also be transported through the cells and into bile. Although e.g. IgA-class antibodies are known to be excreted into bile and later into the GI tract, this pathway of elimination is considered negligible for most biologicals.

Finally, the target-mediated drug disposition (TMDD) can play a significant role. Upon binding to cell-surface targets such as receptors, biologicals can trigger their endocytosis and subsequent intracellular degradation in lysosomes (Vugmeyster *et al.,* 2012). Depending on the physiological distribution of targets, TMDD can be a major elimination step for some biologicals. As it shows how distributing to targets (both on- and off-) to elicit pharmacodynamic effects can be considered an elimination step, it is a prime example of how difficult drawing a line between different ADME events of biologicals can be. Since the number of targets can be limited, this elimination pathway may be saturated even with therapeutic drug concentrations, giving rise to non-linear PK with many biologicals.

Fc receptor binding

The Fc domain in antibodies and biologicals (e.g. mAbs; **Fig. 6a** and engineered Fc-fusion proteins) and the plasma protein albumin is recognized by Fc receptors and complement proteins. While antibody binding to Fc receptors serves biological purposes such as transporting IgGs across the placenta, inducing phagocytosis and microbe killing, it has implications on the PK of both albumin- and Fc-containing biologicals (Sockolosky & Szoka, 2015; Schmidt *et al.,* 2017). Fc-gamma receptors (FcγR) are expressed on the surface of certain phagocytic cells, such as macrophages, and can trigger the intake and subsequent lysosomal degradation of bound proteins (Vugmeyster *et al.,* 2012). The contribution of this pathway of clearance is, however, considered of little significance to many biologicals, as their amounts are often small compared to endogenous antibodies. In fact, only a very small fraction of the full antibody pool, and thus only a small fraction of Fc-containing biologicals, will be

susceptible to clearance via this route (Liu, 2018). Its role cannot however be completely ruled out with all biologicals (Kamath, 2016).

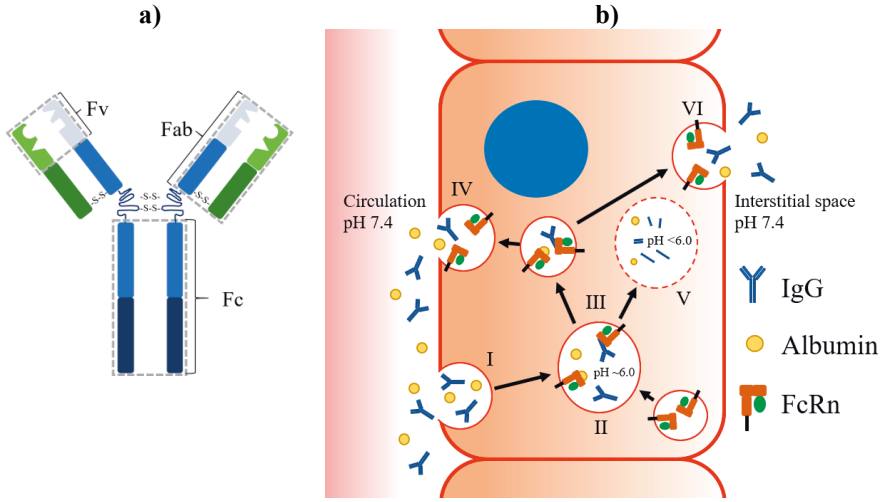

Figure 6. ***a)*** *Schematic diagram of antibody structure and regions. Fv, fragment, variable; Fab, fragment, antigen-binding; Fc, fragment, crystallizable; FcRn, neonatal Fc receptor.* ***b)*** *Model of FcRn's involvement in the transcytosis, and the recycling of albumin and IgGs. Proteins are internalized by pinocytosis or endocytosis (I) and as they are transported along the endosomal pathway, the acidification in the early endosomes results in association with the endosomal FcRn (II). As FcRn-protein complexes and unbound proteins are sorted (III), the FcRn-bound proteins evade lysosomal degradation and are recycled back to the cell membrane and liberated due to low affinity at physiological pH (IV) while unbound proteins are sorted for degradation in the lysosomes (V). In polarized cells the FcRn-mediated transcytosis can direct the FcRn-protein complexes directed to opposing cell membranes, where protein release takes place at physiological pH (VI). Adapted and modified from Roopenian & Akilesh, 2007.*

Unlike with FcγR, binding to the neonatal Fc receptor (FcRn) can significantly impact the PK of biologicals. A major determinant of the binding is its pH dependency; binding to FcRn is negligible at physiological pH but is significantly more pronounced in acidic pH (Roopenian & Akilesh, 2007). While the association between FcRn and Fc fragment is pH-dependent and a prerequisite for the interaction, more distal regions can also affect this interaction (Wang *et al.,* 2011; Jensen *et al.,* 2015; Monnet *et al.,* 2015). It has been recently demonstrated that, e.g., the electrostatic charge of the Fv region of the Fab domain influences the interaction (Schoch *et al.,* 2015; Ternant *et al.,* 2016) and that even very small changes, especially in the complementarity-defining region, can have profound effects on the FcRn binding affinity and thus PK of IgGs (Piche-Nicholas *et al.,* 2018). Furthermore, the impact of regions distal to the Fc domain are evident in the case of Fc-fusion proteins, as they have been shown to exhibit lower binding affinity to FcRn due to the fusion partner (Liu, 2015). Elucidating the molecular basis of FcRn-binding has shed light on the key contributors, i.e., epitopes and residues responsible for the interaction, and will undoubtedly guide the rational design of engineered proteins (Oganesyan *et al.,* 2014; Jensen *et al.,* 2015; Monnet *et al.,* 2015; Sockolosky & Szoka, 2015).

While other factors may influence the binding, their impact on the PK might not be clinically relevant. For example, glycosylation and lack thereof, can have widespread influence on the conformation, function, stability, immunogenicity and PK of biologicals (Goh & Ng, 2018). Although the FcRn-IgG interaction has been

demonstrated to be sensitive to IgG glycosylation *in vitro,* possibly due to altered binding dynamics, this does not seem to translate to changes regarding the *in vivo* PK (Liu *et al.,* 2011; Jensen *et al.,* 2015; Liu, 2015).

FcRn expression is widespread in the body (Pyzik *et al.,* 2015; Latvala *et al.,* 2017), localized mostly in intracellular vesicles, such as endosomes, and to a lesser extent on cell surfaces (Sockolosky & Szoka, 2015). Proteins in circulation are thus thought to be continuously removed mostly through non-specific, fluid-phase pinocytosis and less through receptor-mediated endocytosis (Pyzik *et al.,* 2015; Liu, 2018; **Fig. 6b**: I). Therefore, proteins mostly encounter FcRn in the early endosomes, where upon acidification of the internalized proteins' milieu, association with the FcRn takes place (**Fig. 6b**: II). After sorting, the FcRn-bound proteins avoid lysosomal degradation (**Fig. 6b**: III) and migrate to the cell surface where the FcRn-complexes are dissociated in the physiological pH milieu, thereby releasing the proteins (**Fig. 6b**: IV), whereas unbound proteins are sorted for lysosomal degradation (**Fig. 6b**: V). Furthermore, the FcRn-protein complexes can also be trafficked to opposing cell membranes in polarized cells, such as endothelial cells, and then released due to neutral pH (**Fig. 6b**: VI). This FcRn-mediated transcytosis of proteins is recognized as a mechanism for, e.g., transporting IgGs from the alveoles to systemic circulation, and through the placenta from mother to fetus (Roopenian & Akilesh, 2007).

FcRn-mediated recycling can salvage IgGs and albumin from intracellular clearance and is the main reason why their circulatory half-lives are notably longer compared to other proteins. As such, the impact of binding to FcRn on Fc-containing biologicals' PK is an extended half-life (Shah, 2015). The effect is not only at the systemic level; the local effects of FcRn in different cells and tissues are becoming more evident as its expression is mapped (Latvala *et al.,* 2017; Deissler *et al.,* 2017; Deissler *et al.,* 2018). FcRn's possible role in tissue distribution of biologicals is, however, still under debate as all aspects are not yet fully understood (Kamath, 2016; Shah, 2015). While some report FcRn to have a negligible role in tissue distribution, others have demonstrated FcRn to play a role in the distribution and tissue selectivity of biologicals (Garg & Balthasar, 2007; Chen *et al.,* 2014; Ryman & Meibohm, 2017). Different methodologies, such as physiology-based pharmacokinetic modeling (Ferl *et al.,* 2005; Garg & Balthasar 2007) and both FcRn knock-out (Chen *et al.,* 2014) and humanized FcRn mouse models (Proetzel & Roopenian, 2014; Avery *et al.,* 2016) have been utilized to elucidate FcRn's contribution to the PK of Fc-containing biologicals and to see how modulating this interaction can be utilized in protein (PK) engineering.

2.2.5 Immunogenicity of therapeutic proteins

Therapeutic proteins have a 'self' origin and as such they have little intrinsic toxicity and subsequently rarely cause adverse effects *per se* as they manifest mainly as exaggerated pharmacology (Schellekens, 2010). However, the biggest limitation in their use is that therapeutic proteins are commonly observed to elicit immune responses in patients, i.e., being immunogenic (Descotes & Gouraud, 2008). The immune system can recognize attributes, such as deviations from the native sequence, presence of 'non-self' regions/sequences on the protein, certain epitopes (intrinsic, engineered, and neoepitopes generated via e.g. denaturation), deviations in PTMs such

as glycosylation, and protein aggregates as threatful xenogenic entities that trigger subsequent immune responses (Descotes & Gouraud, 2008; Wadhwa *et al.,* 2015; Kuriakose *et al.,* 2016; Ryman & Meibohm, 2017). Thus, for example, fusion proteins and proteins with mutated sequences, and hence new epitopes, can potentially be more immunogenic than native proteins. Other non-protein factors include, i.a., concomitant therapies, underlying diseases and the route of administration (Schellekens, 2010, Wakshull *et al.,* 2017).

Immunogenicity is a feature of practically all therapeutic proteins. Humanized and even fully human proteins have been shown to induce immune responses, e.g. via protein aggregates (Descotes & Gouraud, 2008; Forooghian *et al.,* 2009; Schellekens, 2010; Deehan *et al.,* 2015; Ryman & Meibohm, 2017). The specific triggers leading to an immune response vary, and the resulting complex coordinated events can involve the recruitment of components of both the adaptive and non-adaptive immune systems (Baker *et al.,* 2010; Deehan *et al.,* 2015; Wadhwa *et al.,* 2015).

Clinical outcomes of therapeutic protein immunogenicity differ. Anti-drug antibodies (ADA), i.e. specific antibodies formed against a (protein) therapeutic or a part of it, can have wide-ranging repercussions on patients. While immune responses frequently lead to only minor pathologies, such as a mild rash, or are completely asymptomatic (Descotes & Gouraud, 2008; Büttel *et al.,* 2011; Smith *et al.,* 2016), in rare cases neutralizing ADAs against therapeutic proteins can cross-react with homologous endogenous proteins, leading to their neutralization and subsequent severe, sometimes even fatal, outcomes (Baker *et al.,* 2010; Deehan *et al.,* 2015; Smith *et al.,* 2016). Fortunately, only a small fraction of patients exhibits undesirable immune responses to biologicals (Kuriakose *et al.,* 2016).

Arguably the biggest impact protein therapeutics elicit via immunogenicity is upon themselves. Due to altered clearance of the affected proteins, subsequent perturbations in their pharmacokinetics and pharmacodynamics occur, mainly manifesting as a lowered efficacy (Smith *et al.,* 2016). The main reasons for halting drug development projects in clinical trial phases II and III are lack of efficacy, safety, and commercial/strategical reasons (Kola & Landis, 2004; Arrowsmith & Miller, 2013). Although safety is less of an issue and a reason for clinical trial discontinuations with therapeutic proteins, lack of efficacy is a frequent hurdle (Hwang *et al.,* 2016; Patel *et al.,* 2017). Since protein immunogenicity is often the root cause for lowered efficacy, and at times also a cause for clinical trial failures (Schellekens, 2010), this highlights the impact it can have on these drugs. To make matters even more complicated, although evaluating therapeutic protein immunogenicity and its clinical significance during drug development is required by regulatory authorities, for example in the European Union and the US (EMA, 2017b; FDA, 2014b), it is highly complex and much of the current methodology is not adequate (Wadhwa *et al.,* 2015). For example, immunogenicity evaluation based on protein structure as well as on most *in vivo* animal models is regarded to be of poor predictive value and thus, unreliable (Deehan, *et al.,* 2015; Smith *et al.,* 2016). Likewise, assessing the local immunogenicity of biologicals, e.g., in the eye, is complicated (Wakshull *et al.,* 2017, Wessels *et al.,* 2018). Therefore, immunogenicity is frequently under-observed during pre-clinical development, becoming evident later in clinical trials or even after years of use in patients. This makes the impact – not only clinical but also financial – of therapeutic protein immunogenicity even more significant.

To recap, biologicals are complex and delicate molecules and thus challenging to produce and handle. On the other hand, compared to traditional small-molecule drugs, biologicals are superior in specificity and elicit fewer adverse effects. From the launch of Humulin® the use of biologicals has since expanded to diverse fields of medicine. Ophthalmology is a field that has seen revolutionary changes with the introduction of biologicals and is at the focus of the following sections of this review.

2.3 The human eye and ocular pharmacotherapy

The eye is a highly complex and specialized organ, not only in its function but also in its physiology (**Fig. 7a**). Anatomically and for drug delivery, the human eye can be divided into anterior and posterior segments, separated by the anterior hyaloid membrane. The anterior segment makes up roughly a third of the eye and consists of the cornea, conjunctiva, iris, ciliary body and the lens, whereas the posterior segment contains the vitreous humor, choroid, retina and optic nerve (Awwad *et al.,* 2017). The tissues of the anterior segment are responsible for focusing and adjusting the amount of light entering the eye, while the conversion of light stimulus to neural signals takes place in the posterior segment and more specifically in the specialized cells of the retina (Delplace *et al.,* 2015).

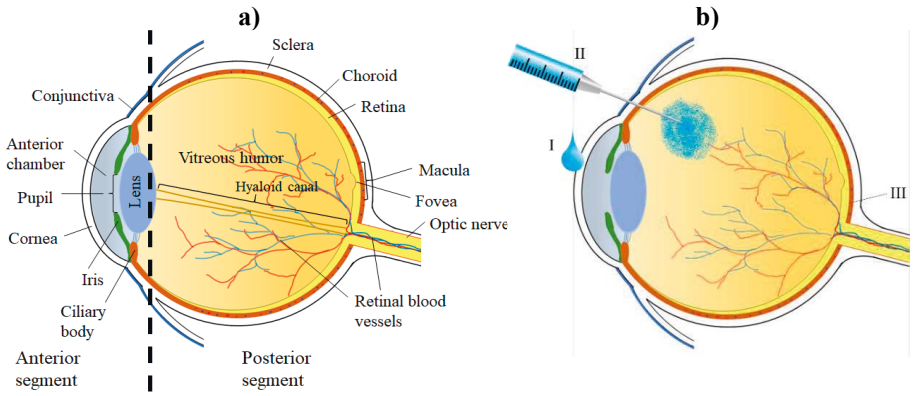

Figure 7. *Schematic diagram of the human eye.* ***a)*** *Anatomical features of the eye,* ***b)*** *selected ocular administration routes: topical (I), intravitreal (II), and systemic (III).*

2.3.1 Ocular pharmacokinetics and administration of therapeutic proteins

The physiology of the eye itself complicates the treatment of posterior segment diseases. Whereas ocular barriers are in place to, for example, prevent pathogen access, they also complicate and prevent the delivery of drugs, including biologicals, into the eye. For brevity, only the factors deemed most important are discussed here and the reader is directed to recent reviews for a more in-depth analysis of ocular pharmacokinetics and limitations therein (del Amo *et al.,* 2017; Awwad *et al.,* 2017; Huang *et al.,* 2018). Furthermore, although the following sections in this thesis discuss ocular pharmacokinetics of biologicals in the intact eye, it should be noted that ocular pharmacokinetics may differ in different disease states (Agrahari *et al.,* 2016).

Topical administration (**Fig. 7b**: I) of eye drops or other dosage forms (e.g. gels), usually by the patients themselves, is the least invasive and most logical way of administering drugs for treating the anterior segment (Wakshull *et al.,* 2017). It is, nonetheless, complicated by several factors: the corneal epithelium is tightly packed and thus limits permeation, while residence time after administration is reduced by blinking, nasolachrymal drainage and tear film turnover, which is often increased due to irritation from the applied drug. Even after a minuscule fraction (< 5%) of a topically administered drug manages to permeate into the anterior chamber, the direction of aqueous humor flow and blood flow of the iris hampers efficient drug distribution past the anterior segment and towards the posterior segment. Low ocular bioavailability and clearance from the anterior chamber result in low drug concentrations in the eye. Therefore, topical administration of small molecule drugs is used only for treating the anterior segment and not the posterior segment of the eye (Ranta & Urtti, 2006; Patel *et al.,* 2013), whereas biologicals are not used at all as topical ocular medications.

Barriers do not only protect the eye from the outside, but also from within the body, as access to the eye from blood circulation is limited by the anterior blood-aqueous barrier (BAB) and the posterior blood-retinal barrier (BRB). These barriers also complicate ocular pharmacotherapy. BAB has two components: endothelium in the iridial and ciliary muscle capillaries, and tight epithelia in the posterior iris and ciliary body (del Amo *et al.,* 2017). The BRB consists of the inner BRB formed by endothelia of the retinal capillaries, and the outer BRB formed by the retinal pigment epithelium (RPE) cells that, for example, provide metabolic and nutritional support to the overlaying photoreceptors (Cunha-Vaz *et al.,* 2011; Dwyer *et al.,* 2011). Both are cellular monolayers with intercellular tight junctions that regulate the permeability of ions, nutrients, proteins and drugs. Whereas smaller molecules can penetrate both blood-ocular barriers, the permeation of biologicals and other macromolecules is effectively stymied mostly due to their large size (El Sanharawi *et al.,* 2010). The permeability of the RPE can be altered due to ageing and in certain pathological conditions, allowing biologicals in the systemic circulation to enter the eye (**Fig. 7b**: III). Interestingly, several publications report clinical improvements in wet AMD patients receiving systemic bevacizumab (Michels, *et al.,* 2005; Moshfeghi *et al.,* 2006; Bolz *et al.,* 2007; Schmid-Kubista *et al.,* 2009). However, these studies were conducted with limited number of patients over relatively short time periods ranging from 3 to 6 months. Whereas they do not report any serious adverse events and thus attest for short-term safety, they do not rule out possible complications associated with long-term systemic bevacizumab administration. Moreover, as these studies did not assess neither aqueous nor vitreal levels of bevacizumab, the underlying mechanism of ocular entry remains somewhat nebulous; entry via an anterior (Bakri *et al.,* 2007a) and/or a posterior route (Magdelaine-Beuzelin *et al.,* 2010) have been suggested. Additionally, it is unclear whether the clinical improvements arise from effects on the choroidal vessels only (El Sanharawi *et al.,* 2010). Hence, to confirm safety and efficacy, more rigorous long-term clinical studies would be required, but this is unlikely as IVT injections (**Fig. 7b**: II) are more efficacious and perceived to be safer than intravenous injections (Moshfeghi *et al.,* 2006). In conclusion, although the ocular entry of biologicals via the systemic circulation is possible and even exploitable, particularly in certain pathologies and acute conditions, it is nevertheless

generally considered unfeasible. The systemic doses required for reaching therapeutic concentrations inside the eye may be disproportionally high and likely to elicit systemic toxicity, limiting the applicability to only carefully screened patients, if not preventing it altogether (Moshfeghi *et al.,* 2006; Schmid-Kubista *et al.,* 2009; El Sanharawi *et al.,* 2010; del Amo *et al.,* 2017).

The most direct means to circumvent these obstacles in treating vitreo-retinal diseases is by delivering drugs via injections, for example as subconjunctivally between the conjunctiva and the sclera or directly in the vitreous as IVT (**Fig. 7b**: II) (Delplace *et al.,* 2015). Although other injection sites and techniques are possible, the IVT injection through the pars plana region has become the method of choice due to certain considerable advantages. Compared to other injection routes and their inherent limitations such as limited injection volume and poor bioavailability, IVT injections result in maximal intraocular bioavailability and effective local drug concentrations in the vitreous, retina and choroid with a reduced dose and minimal systemic side effects (Agrahari *et al.,* 2017; del Amo *et al.,* 2017; Iyer *et al.,* 2019). The drawbacks of this dosing method are, on the other hand noteworthy: as it must be performed by trained medical professionals it is costly, and patients comply poorly as the administration can be uncomfortable and even traumatic (Moreno *et al.,* 2016). The intravitreal half-lives of the administered biologicals – in the range of several days – requires dosing as frequently as every month, and as many compounds have even shorter intravitreal half-lives, they are not clinically acceptable for IVT dosing as they would necessitate even more frequent administration (del Amo *et al.,* 2017; Lau *et al.,* 2018). Clinical trials for comparing different IVT treatments and treatment regimens have been carried out (Awwad *et al.,* 2017). For example, instead of treating based on patient/disease status, adhering to a more rigid treatment frequency was recently shown to correlate with superior treatment outcomes (Wecker *et al.,* 2019). Yet, there appears to be no clear consensus on optimal treatment regimens among clinicians; ophthalmologists often deviate from those recommended while patients can fail to comply, hence the injections are commonly given at longer and possibly therapeutically less optimal intervals (Ferreira *et al.,* 2015; Lanzetta *et al.,* 2017). Furthermore, with every injection there is the possibility of complications and a small but nevertheless real risk of endophtalmitis (Awwad *et al.,* 2017), a sight-threatening condition requiring immediate medical attention. Active pursuits to alleviate these burdens on both the patients and physicians are ongoing. Likewise, there are numerous endeavors in the global research and scientific community to develop new therapies and to improve and enhance available therapies.

Administered biologicals distribute in the vitreous largely by diffusion (del Amo *et al.,* 2017). The transparent vitreous consists mostly of water and has a gel-like structure maintained by a collagen fibril network with anionic glycosaminoglycans such as hyaluronic acid (HA) contributing to the gel viscosity (Meral & Bilgili, 2011; Käsdorf *et al.,* 2015; Patel *et al.,* 2015). This composition is not homogenous and changes as the vitreous undergoes liquefaction during aging. The vitreal diffusion of very large particles is restricted by the collagen fibrils, and the diffusion of cationic smaller species is suppressed by the anionic charges in the biopolymer network (Xu *et al.,* 2013; Käsdorf *et al.,* 2015). Therefore, overall diffusion restrictions to smaller neutral and anionic species are minor. However, it remains unclear whether the anionic network affects the mobility of biologicals with cationic regions and if this has clinical

significance (del Amo *et al.,* 2017). Nevertheless, vitreal diffusion is not considered to impede the diffusion of biologicals to the retina, as observed with a porcine *ex vivo* model with heat shock protein 70 (Subrizi *et al.,* 2014). Yet, more specific interactions with vitreal components may retard the vitreal diffusion of biologicals and could hence be utilized for extending their vitreal half-lives (Fuchs & Igney, 2017; Ghosh *et al.,* 2017; Rimpelä *et al.,* 2018).

Upon intravitreal administration, biologicals are abruptly subjected to a new, possibly destabilizing environment; the stabilizing formulation components are effectively and swiftly diluted in the vitreous and thus cease providing stabilization to the proteins (Patel *et al.,* 2015; del Amo *et al.,* 2017). Unprotected exposure to the physiological temperature, proteolytic enzymes and other vitreal components may have a destabilizing effect on biologicals, potentially leading to degradation and aggregation during the residence time in the vitreous (Patel *et al.,* 2015; Patel *et al.,* 2017). Due to challenges associated with collection of clinical samples, the vitreal stability of therapeutic proteins is understudied and instead carried out with surrogate models. For example, in a study utilizing *ex vivo* porcine vitreous model, shifts in pH were observed to destabilize proteins (Patel *et al.,* 2015). Conversely, the buffering capacity of sampled human vitreous was demonstrated to resist pH shifts and even maintain a stable pH upon administration of clinically used doses of anti-vascular endothelial growth factor (VEGF) biologicals (Sobolewska *et al.,* 2017). Although this was postulated to reflect the *in vivo* stability, it is nonetheless possible that current *ex vivo* and animal models do not accurately represent the *in vivo* situation in human eyes. Recently, a more practical and feasible approach was described as the *in vivo* stability of IVT injected lampalizumab and its variants was evaluated using aqueous humor samples collected from patients (Loyet *et al.,* 2019). Nonetheless, although the eye is commonly described as an immune privileged organ, as local immune responses to IVT administered biologicals have been reported (Wessels *et al.,* 2018), it clearly is not immune incompetent. Therefore, it is possible, although not yet established, that protein destabilization and unfolding contribute to local immunogenicity after IVT administration. Clearly, many questions still need answers before reaching a comprehensive understanding of the influencing factors and phenomena, and how intravitreal stability of biologicals translates into potential changes in PK, toxicity and efficacy.

The retinal entry and transretinal migration of biologicals depend on factors such as molecular weight, structure, charge and lipophilicity (El Sanharawi *et al.,* 2010; Balaratnasingam *et al.,* 2015; del Amo *et al.,* 2017). The first barrier upon IVT injection of a biological is to penetrate the inner limiting membrane (ILM), the retinal layer at the retina's vitreal border. The thickness of ILM varies topographically, during aging and in certain disease states, although the impact of these changes is yet to be investigated (El Sanharawi *et al.,* 2010; del Amo *et al.,* 2017). In studies with FITC-dextrans of various molecular weights (M_r), the retinal exclusion limit, i.e. the maximal size of particles capable of retinal penetration, was determined as 76 kDa and 100 kDa with fixed and unfixed human retinal tissues, respectively (Jackson *et al.,* 2003). There are indications of similar as well as larger M_r thresholds resisting transretinal migration in different layers of the retina (El Sanharawi *et al.,* 2010; del Amo *et al.,* 2017).

Retinal penetration studies with multiple biologicals and macromolecules in several different animals have yielded somewhat conflicting results (El Sanharawi *et*

al., 2010). One of the earliest studies supported M_r being a major factor as a Fab fragment was observed to diffuse through the whole retina, whereas the larger, full-size mAb trastuzumab failed to penetrate even the ILM (Mordenti *et al.,* 1999). Bevacizumab (a full-sized mAb; 150 kDa) was however demonstrated to penetrate the full thickness of the retina in rabbits (Shahar *et al.,* 2006), mice (Kim *et al.,* 2009), and cynomolgus monkeys (Heiduschka *et al.,* 2007). Similarly, retinal penetration has also been demonstrated, e.g., with ranibizumab (a Fab-fragment; 48 kDa) in rabbits (Gaudreault *et al.,* 2007) and rhesus monkeys (Mordenti *et al.,* 1999), and with ziv-aflibercept (VEGFR-2 Fc fusion protein; 115 kDa) in rabbits (Ramon *et al.,* 2018). Hence, observations of molecules larger than proposed diffusion limits nonetheless penetrating the retina have evoked discussion of other mechanisms, including active transport mechanisms (Eng & Kertes, 2006; Heiduschka *et al.,* 2007; Terasaki *et al.,* 2015). Since dissimilar penetration has been observed with biologicals of equal sizes, the possibility of the biologicals' target specificities playing a role cannot be ruled out (Shahar *et al.,* 2006; Heiduschka *et al.,* 2007; El Sanharawi *et al.,* 2010). Furthermore, as bevacizumab was shown to distribute the full thickness of the retina after a high IVT dose, it has also been proposed that higher doses could overwhelm the potential diffusion barriers and that retinal permeation might hence be related to dose (Shahar *et al.,* 2006). Similar results were seen after IVT administration of high doses of pooled human IgGs in rabbit eyes (Han, 2004). The proposed dose-dependency on retinal penetration could be clinically relevant since commonly used doses of anti-VEGF biologicals are more than enough to exhaust free vitreal VEGF (Stewart 2018a), however, there currently seems to be little experimental evidence to support this. Although poorly investigated, the influence of charge has also been speculated as the dissimilar retinal penetration of tissue plasminogen activator and its analogue tenecteplase was attributed to their charge differences (Kwan *et al.,* 2006).

Research on the retinal penetration of biologicals has predominantly been done with more qualitative methods, e.g., immunohistochemistry and microautoradiography, while truly quantitative estimates of the retinal penetration and distribution of biologicals are sparse. Since these studies have commonly been done with animal eyes, the translation to (diseased) human eyes remains somewhat vague (Eng & Kertes, 2006). Furthermore, whether penetrating the full thickness of the retina is required for achieving clinical efficacy is not fully established, as this may differ depending on different biologicals' modes and sites of action (Eng & Kertes, 2006; del Amo *et al.,* 2017; Mehta *et al.,* 2019). Although the rationale for developing smaller biologicals, such as the anti-VEGF agents ranibizumab and brolucizumab, is partially based on the assumption that a smaller size is favorable for efficient retinal penetration (Ferrara *et al.,* 2007; Magdelaine-Beuzelin *et al.,* 2010; Browning *et al.,* 2012), this, however, seems to be mostly hypothetical and not necessarily experimentally supported. There is clearly a need for comprehensive and systematic studies on the factors influencing retinal penetration of biologicals (Hutton-Smith *et al.,* 2017) and for the development of new methodologies.

As biologicals permeate poorly through the BAB and the BRB, their vitreal clearance is considered to take place primarily via the anterior route, i.e., by diffusion to the anterior segment and subsequent removal with the aqueous humor outflow to Schlemm's canal (Shatz *et al.,* 2016; del Amo *et al.,* 2017; Awwad *et al.,* 2017). This has been observed with, e.g., ranibizumab (Krohne *et al.,* 2012) and lampalizumab

(Loyet *et al.,* 2019) and is further supported by several PK modelling studies (Hutton-Smith *et al.,* 2016; del Amo *et al.,* 2017; Hutton-Smith *et al.,* 2017; Lamminsalo *et al.,* 2018; Rimpelä *et al.,* 2018). Intravitreal half-life of biologicals correlates strongly with the hydrodynamic size but less strongly with the molecular weight of the biological, as only increases in hydrodynamic size caused a linear increase in vitreal half-life due to resulting differences in molecular diffusivity (Gadkar *et al.,* 2015; Shatz *et al.,* 2016; Rimpelä *et al.,* 2018; Crowell *et al.,* 2019).

Other clearance mechanisms and elimination routes have been proposed and described in the literature. While there is some support for the role of posterior clearance pathways (Gaudreault *et al.,* 2005; Heiduschka *et al.,* 2007; Krohne *et al.,* 2012), their contribution remains unclear and their significance is nonetheless currently considered low when compared to elimination via the anterior route (Gadkar *et al.,* 2015; del Amo *et al.,* 2017; Deissler *et al.,* 2017; Mehta *et al.,* 2019).

The role of FcRn-mediated recycling and transcytosis on ocular drug disposition of biologicals has been widely speculated but remains controversial. Ocular expression of FcRn in humans has been observed in several locations, e.g., in the retina, ciliary body, the vascular components of the BRB and the RPE (van Bilsen *et al.,* 2011; Krohne *et al.,* 2012; Powner *et al.,* 2014). Furthermore, FcRn expression may be upregulated in certain disease states (Kim *et al.,* 2009; Dithmer *et al.* 2016). FcRn-mediated posterior clearance has been speculated to account for the surprisingly small ocular half-life difference between bevacizumab, (a full-size mAb, 150 kDa), and ranibizumab, (a Fab fragment 48 kDa) (Krohne *et al.,* 2012). Fc-containing biologicals were demonstrated to be taken up by human RPE and primary porcine RPE cells *in vitro* whereas this uptake was inhibited with an anti-FcRn antibody (Dithmer *et al.,* 2016). In the same study, transport was observed with a porcine RPE-choroid organ explant culture, corroborating the role of FcRn in the recycling and transcytosis of biologicals in the posterior eye segment (Dithmer *et al.,* 2016). Accordingly, IVT administered bevacizumab penetrated the retina and was eliminated into the systemic circulation through the BRB in wild-type mice but not in FcRn knockout mice (Kim *et al.,* 2009). Furthermore, in the same *in vivo* study by Kim et al (2009), posterior clearance of bevacizumab was more rapid in laser-photocoagulated rat retinas, concluded to be a result of upregulated FcRn expression. *Vis-à-vis*, the role of FcRn in mediating the ocular entry from the systemic circulation has been proposed, e.g. with aflibercept and bevacizumab (Magdelaine-Beuzelin *et al.,* 2010). After IVT injections, small amounts of bevacizumab (Bakri *et al.,* 2007a), but not ranibizumab (Bakri *et al.,* 2007b), were detected in the serum and the fellow uninjected eye in rabbits. Intriguingly, after bevacizumab injection in only one eye, regression of neovascularization was also observed in uninjected fellow eyes of some diabetic retinopathy patients, with the ocular entry hypothesized to be mediated by FcRn (Avery *et al.,* 2006). Although FcRn has been speculated to have a role in the ocular entry, distribution, and clearance of biologicals, it has also been argued to be only a minor or even insignificant contributor in the vitreal half-life of biologicals (Mehta *et al.,* 2019), with the issue yet to be conclusively elucidated.

Whether the FcRn-binding plays an equal role in the PK of native Fc-containing proteins and Fc-fusion proteins has been speculated (Dithmer *et al.,* 2016). *In vitro* studies with retinal endothelial cells (REC), cells lining the retinal capillaries and responsible for the inner BRB, have shown FcRn-mediated recycling and transcytosis

of bevacizumab and aflibercept (Deissler *et al.,* 2016; Deissler *et al.,* 2017). A follow-up *in vitro* study concluded that a fraction of uptaken aflibercept is destined for lysosomal degradation unlike bevacizumab, indicating a dissimilar fate between IgG and Fc fusion proteins (Deissler *et al.,* 2018). An *in vivo* study in rabbits demonstrated that annulling FcRn-binding by changing the Fc-domain to a coiled-coil domain on a VEGF-Trap fusion protein prolonged vitreal and retina/choroid half-lives by 39% and 130%, respectively, owing to diminished FcRn-mediated elimination (Joo *et al.,* 2017). These findings support the hypothesis that as the FcRn acts as an efflux transporter, the presence of Fc domain would be a crucial determinant in the disposition of intravitreal biologicals (Kim *et al.,* 2009). On the other hand, they are in contrast with an *in vivo* study demonstrating that, regardless of its capacity for FcRn-binding, Fc alone had Fab-like ocular PK (Gadkar *et al.,* 2015). Furthermore, as the ocular PK of a null IgG (an IgG with abolished Fc receptor binding) was similar to that of an intact IgG, the study concluded FcRn binding to have little clinical impact on ocular PK in comparison to systemic PK, possibly due to modest levels of FcRn expression in the eye.

2.3.2 Diseases of the posterior eye segment

Diseases of the posterior segment tissues are the leading cause of visual impairment and blindness worldwide (Awwad *et al.,* 2017). Despite these diseases manifesting with somewhat similar symptoms, they affect different cell types (del Amo *et al.,* 2017). Although e.g. inflammation has been recognized in the aging retina (Chen *et al.,* 2010) and as a central component of the underlying pathogenesis in many retinal diseases (Pascual-Camps *et al.,* 2014), various other factors contribute to each disease. Nonetheless, as many of these diseases are age-related, their incidences are projected to increase in the following decades due to increased life expectancy (Delplace *et al.,* 2015). This poses as a source of significant pressure on healthcare systems worldwide.

Age-related macular degeneration (AMD) is currently the most frequent cause of legal blindness among the elderly populace in the developed world (Kinnunen *et al.,* 2012; Ghasemi *et al.,* 2018). While peripheral vision remains unaffected, AMD leads to progressive loss of central vision. The etiology of this disease is still not fully understood, but multiple factors such as age, genetic predisposition, smoking, and other environmental causes contribute to the pathogenesis (Kinnunen *et al.,* 2012; Volz & Pauly, 2015; Kamao *et al.,* 2018). Early AMD is characterized by the formation of drusen, i.e., deposits of cellular debris between the choroid and RPE, that restrict nutrient supply to the RPE and overlying photoreceptors (Volz & Pauly, 2015; Bandello *et al.,* 2017). Advanced AMD is clinically classified in two distinguished forms: the dry form and the more severe and faster progressing exudative wet form (10-20% of patients) (Kinnunen *et al.,* 2012; Dolgin 2017); although distinct in pathophysiology, wet AMD often progresses from (early) dry AMD (Delplace *et al.,* 2015; Dolgin, 2017). The formation of drusen remains the main characteristic in dry AMD, with the associated impairment of nutrient supply eventually leading to large areas of photoreceptor and RPE atrophy in advanced dry AMD, also known as geographic atrophy (GA). The hallmark of wet AMD, and the distinguishing feature between the two forms, is choroidal neovascularization (CNV), i.e., the formation of new and abnormal blood vessels in the choroid. The growth of these vessels is

triggered by hypoxic conditions and driven by the RPE's overexpression and release of pro-angiogenic factors, most importantly VEGF (Kinnunen *et al.,* 2012; Delplace *et al.,* 2015). These new vessels extending from the choroid to the RPE and retina are fragile and fenestrated, resulting in RPE and even retinal detachment due to underlying fluid leakage and hemorrhaging, eventually leading to cell death and loss of vision (Peng *et al.,* 2010; Volz & Pauly, 2015). Currently, only wet AMD can be treated with pharmaceuticals as several candidates for preventing RPE and photoreceptor damage in GA have failed in clinical trials, making the progression of dry AMD so far unstoppable (Bandello *et al.,* 2017).

The prevalence of both type I and type II diabetes is projected to increase dramatically in the following decades, along with diabetes-associated complications. Diabetic retinopathy (DR) is the most common complication and microvascular event of diabetes, and the most common cause of vision loss in working-age individuals (Duh *et al.,* 2017; Wang & Lo*,* 2018); out of the nearly 300 million diabetics worldwide, over a third are estimated to have DR and in a third of these cases, the condition is sight-threatening (Gardner & Chew, 2016). Early stage DR is categorized to be non-proliferative (NPDR), whereas proliferative DR (PDR) is typical in the advanced stage. During NPDR the patients can be asymptomatic, while significant vision loss can be seen in PDR patients (Duh *et al.,* 2017; Wang & Lo, 2018). Overall, by disturbing multiple biochemical processes, hyperglycemia damages blood vessels in the retina (Stewart 2017; Wang & Lo, 2018). Increased vascular permeability and capillary occlusion/ degeneration during NPDR manifest as characteristic pathologies, such as cotton wool spots, intraretinal hemorrhages, and microaneurysms (Duh *et al.,* 2017; Wang & Lo, 2018). Gradually worsening non-perfusion during NPDR leads to ischemia and subsequent hypoxia, triggering an angiogenic response, e.g., via VEGF upregulation. The pro-angiogenic factors stimulate neovascularization in the preretinal space, the defining characteristic of PDR. Like in wet AMD, the newly formed blood vessels are aberrant and can in severe cases burst, resulting in significant hemorrhaging into the vitreous, effectively clouding the patient's vision. Moreover, DR patients can develop a pathologic complication called diabetic macular edema (DME), where due to the breakdown of BRB, leaking fluids and proteins accumulate sub- and intraretinally (Stewart 2017; Wang & Lo, 2018). Macular swelling due to this fluid build-up is a frequent occurrence and DME-associated vision impairment is the most common cause of vision loss in DR patients. While glycemic control is the firm basis for diabetes treatment and thus preventing the occurrence and progression of DR, anti-VEGF treatments against neovascularization (in PDR) and changes in vascular permeability (in DME) are also used (Gardner & Chew, 2016).

Glaucoma affects 70-80 million people globally and is the second most common cause for blindness (Foldvari & Chen, 2016). Glaucoma is a term used to describe a collection of multifactorial neurodegenerative diseases where, regardless of mechanisms and pathways, the characteristic degeneration and death of retinal ganglion cells (RGC) leads to optic nerve damage and progressive and permanent vision loss. Elevated intraocular pressure (IOP) is the main associated risk factor and while symptomatic glaucoma management is primarily carried out with IOP-lowering pharmacotherapy, IOP control in some patients is challenging, while other patients experience RGC impairment even at normal IOP (Johnson *et al.,* 2011; García-Caballero *et al.,* 2017). Since symptomatic treatment of glaucoma does not always

translate into salvaging retinal function, direct neuroprotection has been proposed to prevent visual impairment, especially since the pathogenesis of glaucoma has been linked to deficiencies of certain neurotrophic factors (Johnson *et al.,* 2011; Foldvari & Chen, 2016).

Retinitis pigmentosa (RP) is a class of inherited retinal dystrophies featuring gradual degeneration and loss of photoreceptors. RP has a global prevalence of approximately 1:4000 and exhibits clinical and genetic heterogeneity; the genetic predisposition to RP is complex as the contribution of more than 100 mutations in over 40 genes have been linked to the condition, although the genotypes of most patients are unknown (Hartong *et al.,* 2006; Ferrari *et al.,* 2011; Birch *et al.,* 2013). In most cases the outer retina alone is initially affected; the outer retina has few cone photoreceptors compared to rod photoreceptors, with the former responsible for color and fine vision and the latter accounting for scotopic vision. Therefore, due to predominant degeneration of rods, patients typically exhibit loss of night vision, as well as loss of peripheral vision experienced as narrowing of the visual field, i.e., 'tunnel vision'. RP can advance toward the macula, where cone degeneration can eventually result in central vision impairment and even complete blindness. In the atypical cone-rod dystrophy the sequence of events is reversed as cone degeneration takes place first. Unfortunately, there are no established, reliable treatments for preventing the progress of RP or restoring vision (Sahni *et al.,* 2011; Zheng *et al.,* 2015). Although the recent approval of voretigene neparvovec, a gene therapy for RPE65 mutation-associated RP patients is a notable milestone, the identification of specific mutations is a prerequisite for genetic strategies, making them inherently limited in applicability (Sahni *et al.,* 2011; Ameri, 2018). The slow disease progression and the multitude of underlying biochemical abnormalities present considerable challenges, and a 'universal' treatment for all RP forms and stages would need to be independent of the degeneration etiology (Hartong *et al.,* 2006; Birch *et al.,* 2013; Zheng *et al.,* 2015). Such pan-RP treatment strategies have so far been proposed and explored albeit with varying results; using anti-apoptotic factors and neurotrophic factors to prevent photoreceptor cell death and providing long-term neuroprotection are considered broadly applicable, since photoreceptor degeneration is present in all cases regardless of etiology. Other approaches such as mechanism-specific gene therapies, general neuroprotection via gene therapy, as well as regenerative therapies have been and are currently being explored (Sahni *et al.,* 2011; Lipinski *et al.,* 2015; Zheng *et al.,* 2015).

Other chronic and acute diseases of the posterior segment – some of which also lead to visual impairment – include retinal vein occlusion and inflammatory and infectious conditions, such as posterior uveitis and cytomegalovirus retinitis (Thrimawithana *et al.,* 2011; Agrahari *et al.,* 2017; Awwad *et al.,* 2017). Treatments in these conditions depend for instance on the symptoms and involved pathogens and include antimicrobial, antiviral, and anti-inflammatory agents administered intravitreally as injections and implants.

2.4 Biologicals in posterior segment diseases – Eyeing the future

Small molecule drugs are used to combat for instance inflammation and elevated intraocular pressure in the eye. Aside from these treatments, certain diseases of the

posterior eye segment currently rely on biologicals as the cornerstone of their management. Although biologicals have longer intravitreal half-lives than small-molecule drugs, they still require administration by IVT injections on a monthly to trimonthly basis. Taking into account the invasiveness of intravitreal injections, the current situation is far removed from ideal, and there is a clear and urgent call for strategies to provide true long-term clinical improvements to patients without frequent and invasive administration. Different pharmacologic modalities as well as varying approaches in drug delivery, controlled release, half-life extension, as well as alternative administration routes and combinatorial approaches are being actively explored (del Amo *et al.,* 2017, Arranz-Romera *et al.,* 2019). However, these efforts are yet to yield a truly marked improvement in efficacy or to be realized as a product on the market (Delplace *et al.,* 2015; Iyer *et al.,* 2019). The following sections review the available biologicals as well as some of the currently studied and developed novel modalities and drug delivery approaches.

2.4.1 Anti-VEGF strategies

Since the recognition of VEGF-mediated angiogenesis as part of the pathology in, e.g., wet AMD and DR, the VEGF-pathway has been exploited as a target of pharmacotherapy. Although there is off-label use of biologicals in certain (acute) ocular conditions, all currently approved biologicals for ocular indications administered via intravitreal injection target the VEGF-pathway (Radhakrishnan *et al.,* 2017; Mandal *et al.,* 2018) and are listed in **Table 3**. Also, some of the plentiful therapeutic proteins in development for posterior segment diseases as of writing are listed in the Appendix as **Table S1**.

Table 3. *Biologicals used in neovascular diseases of the retina (adapted and modified from Zhang et al., 2015; de Oliveira Dias et al., 2016; Radhakrishnan et al., 2017; Al-Khersan et al., 2019).*

Biological	**Structure**	**Target specificity/ mechanism**	**Ophthalmic indications**
Bevacizumab (Avastin®)	Full-size humanized mAb	Binds all VEGF-A and VEGF-B isoforms	* wet AMD DME
Ranibizumab (Lucentis®)	Humanized Fab fragment	Binds all VEGF-A isoforms	wet AMD DME
Aflibercept (Eylea®)	2nd Ig domain of VEGFR-1 and 3rd Ig domain of VEGFR-2 fused to human IgG1 Fc	VEGF-Decoy; all VEGF-A and VEGF-B isoforms, PlGF	wet AMD DME
Conbercept† (Lumitin®)	2nd Ig domain of VEGFR-1 and 3rd & 4th Ig domains of VEGFR-2 fused to human IgG1 Fc	VEGF-Decoy; all VEGF-A, VEGF-B and VEGF-C isoforms, PlGF	wet AMD DME
Pegaptanib (Macugen)	PEGylated RNA aptamer	Binds VEGF-A_{165}	wet AMD
Brolucizumab (Beovu®)	Humanized ScFv fragment	Binds all VEGF-A isoforms	wet AMD

AMD, age-related macular degeneration; DME, diabetic macular edema; Fab, fragment, antigen-binding; Fc, fragment, crystallizable; mAb, monoclonal antibody; PEG, polyethylene glycol; PlGF: Placental growth factor; ScFv, single-chain variable fragment; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor. *Off-label uses. † Approved in China; currently in phase III clinical trials in USA.

Other biologicals targeting the VEGF-pathway have demonstrated potential as treatments in neovascular posterior segment diseases, and many are currently in clinical trials (Stewart, 2017; Stewart, 2018b). OPT-302, a VEGF-C and VEGF-D

binding fusion protein, is currently in phase II clinical trials for wet AMD in combination with ranibizumab, as well as in phase I trials for DME in combination with aflibercept (Mandal *et al.,* 2018; Opthea Ltd., 2018; Opthea Ltd., 2019). Instead of binding and capturing free intravitreous VEGF, blocking the VEGF receptor (VEGFR) and hence inhibiting receptor binding is a potential and intriguing strategy. Tanibirumab, a full-sized mAb targeting VEGFR-2 suppressed laser-induced CNV in a rat model (Kim *et al.,* 2014). Ramucirumab (Cyramza®), another anti-VEGFR-2 mAb currently approved for treating metastatic colorectal cancer is also of interest in controlling neovascularization in the retina (Fleetwood *et al.,* 2014). However, as of 2016 no clinical programs to study this had been initiated (Falcon *et al.,* 2016). Another approach – with possible applications in developing biologicals in general - explores the benefits of enhancing VEGF binding. To allow for less frequent intravitreal administration by increasing the VEGF binding potency and capacity over, for example, ranibizumab, a VEGF dual domain antibody (dAb) was developed (Walker *et al.,* 2016). These enhancements did indeed translate into enhanced potency using *in vitro* angiogenesis models. Lastly, binding of VEGF-A to its cognate receptor was inhibited *in vitro* with Affibodies, a class of small (~6 kDa) and robust proteins, that were engineered to target VEGFR-2 with high affinity (Fleetwood *et al.,* 2014). Whereas they are amenable for production in prokaryotic hosts as well as by chemical peptide synthesis, they are also exciting as anti-angiogenic candidates owing to their considerably small size that may allow them to be applied via alternative administration routes, including topically as eye drops (Fleetwood *et al.,* 2016), although their retinal bioavailability can be expected to be poor given that distributing into the vitreous and retina in clinically significant concentrations is yet to be demonstrated.

While many biologicals are yet to be evaluated in clinical studies for ocular indications, there are interesting new therapeutic proteins just 'outside the field of vision'. Brolucizumab, an anti-VEGF-A single-chain antibody fragment demonstrated efficacy in treating wet AMD in two completed phase III clinical trials (Iyer *et al.,* 2019) and has recently gained approval as a treatment for wet AMD from the FDA (Markham, 2019). Abicipar pegol, a PEGylated small protein targeting VEGF-A and the first candidate in the emerging class of antibody mimetics termed the designed ankyrin repeating protein (DARPin®) has also shown marked potential (Plückthun, 2015). Results from phase II trials raised expectation that were later met in phase III trials as abicipar demonstrated non-inferiority to ranibizumab with a longer intravitreal half-life, allowing for a less frequent dosing for treating wet AMD and DME; a marketing license application was filed to FDA in 2019 (Allergan/Molecular Partners, 2018; Callanan *et al.,* 2018). With all this said it should, however, be noted that the rationale of pan-VEGF targeting has been called into question. VEGFs and VEGF isoforms differ in their receptor binding, subsequently triggering signaling of varying effects *in vivo* (Apte *et al.,* 2019). Moreover, some VEGF isoforms are pro-angiogenic whereas others are anti-angiogenic and neuroprotective, thus making inhibition of the latter undesirable (Amadio *et al.,* 2016).

Some patients fail to respond to anti-VEGF therapies while others may experience initial improvements but become less responsive and even resistant as treatment progresses (Forooghian *et al.,* 2009; Yang *et al.,* 2016; Cabral *et al.,* 2017). Resistance to anti-VEGF therapies is postulated to stem from, e.g. other pathogenic

pathways, the induction of other compensatory angiogenic mechanisms, as well as upregulation of VEGF production in macrophages (Yang *et al.,* 2016). Although the clinical efficacy of anti-VEGF drugs has not been reported to be affected by immunogenicity, a recently described method was capable of detecting and confirming the presence of intravitreal anti-drug antibodies (ADAs) (Wakshull *et al.,* 2017; Wessels *et al.,* 2018); neutralization of anti-VEGF biologicals in the vitreous could be (partially) responsible for the development of resistance. Entry of ADAs from the systemic circulation is also possible since posterior ocular barriers may get compromised in different pathologies, allowing the entry of macromolecules (Moshfeghi *et al.,* 2006).

2.4.2 Non-VEGF targeting modalities

As of early 2020, biologicals targeting VEGF are the only effective and approved pharmaceuticals available for treating certain posterior eye segment diseases, and the vast potential of utilizing other targeting modalities is yet to bear tangible fruit. Given the multifactorial and complex nature of these diseases it is clear that novel biologicals with diverse modes of action are in high demand and that for better outcomes, multimodal approaches, i.e., utilizing combinatory therapies, may be required (Arranz-Romera *et al.,* 2019). As our understanding of the pathogenesis of posterior segment diseases increases, intense research on utilizing potential new targets is currently underway, surely to be followed by the development of new therapies (Shatz *et al.,* 2018).

Many different approaches are being studied, and the pipeline of novel pharmaceuticals for various ocular indications features biologicals with different modalities along with numerous ‘unique-in-class’ molecules as well. While the pipeline does include e.g. small-molecules, gene therapies, and aptamers, protein-based biologicals are at the forefront and are the focus of this thesis. Moreover, the examples presented in the following chapters are in no way comprehensive. Recent publications provide more thorough summaries on new anti-angiogenic therapies as well as on other completely new and emerging modalities and strategies to treat posterior segment diseases (Volz & Pauly, 2015; Bandello *et al.,* 2017; Cabral *et al.,* 2017; Radhakrishnan *et al.,* 2017; Mandal *et al.,* 2018; Stewart 2018b; Yerramothu, 2018).

Targeting other angiogenic pathways

Aside from VEGF, many other factors and pathways are involved in angiogenesis and neovascularization, and therapeutic strategies to block or enhance them may offer more efficient control/inhibition of dysregulated angiogenesis in posterior segment diseases (Tombran-Tink & Barnstable, 2003a; Yang *et al.,* 2016; Chakravarthy *et al.,* 2017; Wang & Lo, 2018).

Pigment epithelium-derived factor (PEDF), is a multi-functional glycoprotein, and as one of the most potent anti-angiogenic factors also known for its neurotrophic activities (Tombran-Tink & Barnstable, 2003a & 2003b). In the eye, PEDF is thought to counteract the actions of VEGF, with neovascularization kept in balance through maintaining an equilibrium between these two factors (Cabral *et al.,* 2017). This hypothesis is supported by observations on decreased PEDF levels, and hence

disrupted equilibrium, in several posterior segment diseases (Gvritishvili *et al.,* 2010). The role of PEDF in AMD pathogenesis has received support as the connection between PEDF gene polymorphisms and AMD occurrence in northern Chinese populations was demonstrated recently (Hao *et al.,* 2018). Although the effectivity of ocular PEDF treatment has been confirmed, for example, in an animal model of neovascularization, studies to validate this in humans are direly needed to bring the protein into clinical use (Cabral *et al.,* 2017).

Endoglin is a protein overexpressed in proliferating endothelial cells and essential for angiogenesis (Kaplon & Reichert, 2018). Carotuximab, a chimeric antibody targeting endoglin, was developed as an anti-angiogenesis treatment and is currently in multiple clinical trials in patients with varying cancer types. Furthermore, an ophthalmic formulation of carotuximab is under development and currently undergoing phase II clinical trials in patients with wet AMD as a co-therapy with ranibizumab (Tracon Pharma, 2018).

Integrins are transmembrane adhesion proteins responsible for attaching cells to their surrounding matrix via ligands such as fibronectin, collagen and laminin (Ishikawa *et al.,* 2015; Cabral *et al.,* 2017). In wet AMD, among other integrins, $\alpha_5\beta_1$-integrins are expressed around neovascular tissues (Volz & Pauly, 2015). By disrupting the interaction between fibronectin and $\alpha_5\beta_1$-integrin, the chimeric antibody volociximab mediates a pro-apoptotic effect on the proliferating vascular endothelial cells, in essence acting as an angiogenesis inhibitor. Volociximab underwent phase I studies in combination with ranibizumab in patients with wet AMD, however, the study was terminated, and only limited results have been published (Ishikawa *et al.,* 2015; Volz & Pauly, 2015). Although phase II studies were planned, the developing company has shifted their focus and no further clinical trials with ocular volociximab are underway.

Elevated levels of vitreal insulin-like growth factor-1 (IGF-1) and insulin-like growth factor-2 (IGF-2) have been observed in PDR and AMD patients, respectively (Agarwal *et al.,* 2015, Cabral *et al.,* 2017). The overexpression of intraocular IGF-1 has been linked to BRB disruption, increased vascular permeability and both retinal and choroidal neovascularization, whereas IGF-2 stimulates angiogenesis, making their inhibition an attractive approach for treating DME and DR. A strategy of intravenously administered human mAb teprotumumab, an IGF-1 receptor antagonist, takes advantage of this pathologic situation that permits biologicals to enter from the circulation and hence also elicit their actions *in oculo*, and is currently in an open-label phase I study in patients with DME (Agarwal *et al.,* 2015; Stewart 2017).

Sphingolipids are bioactive lipids recognized as signaling molecules participating in a variety of both physiological and pathophysiological processes (Volz & Pauly, 2015). Increasing evidence points to sphingosine-1-phosphate (S1P) being involved in modulating the inflammation and neovascularization associated with wet AMD, and also affecting the production of VEGF and other angiogenic factors, in other words having both direct and indirect regulatory roles in retinal angiogenesis (Sabbadini, 2011). The utility of anti-S1P mAbs has been displayed in an *in vivo* CNV model and an ocular formulation of the humanized murine-derived anti-S1P mAb sonepcizumab, a.k.a. iSONEP™, was developed for treating wet AMD and possibly other ocular disorders. Following successful phase I safety studies (Stoller *et al.,* 2010), sonepcizumab, however, did not provide adequate improvements over

ranibizumab in the completed phase II study either as a monotherapy or in combination with ranibizumab (Lpath Inc. 2015; Lpath Inc. 2016). This drug candidate is no longer in clinical development.

Angiopoietins (ANG), a class of vascular growth factor proteins that among their other biological roles function in regulating vascular development and permeability (Wang & Lo, 2018). While ANG-1 stabilizes vasculature and inhibits CNV, ANG-2 induces fluid leakage by destabilizing the vasculature and acts synergistically with VEGF by rendering the endothelial cells more responsive to VEGF and other pro-angiogenic factors (Chakravarthy *et al.,* 2017). ANG-2 is upregulated in neoangiogenic diseases, hence counteracting its function could be a viable therapeutic strategy in treating neovascular diseases of the retina. Nesvacumab is an anti-ANG-2 human mAb that, through ANG-2 inhibition, decreases vascular permeability (Wang & Lo, 2018). It did not provide sufficient efficacy in combination with aflibercept in wet AMD and DME patients, failing to proceed past phase II trials. Other anti-ANG biologicals are nonetheless in development. The preclinical stage trispecific Nanobody® BI-X is engineered to bind VEGF, ANG-2 and albumin (Fuchs & Igney, 2017) whereas faricimab (RG7716) is a bispecific mAb against VEGF-A and ANG-2 (Regula *et al.,* 2016). Generated with CrossMAb technology, faricimab has been further engineered to optimize its ocular PK by abolishing binding interactions with FcγR and FcRn (Klein *et al.,* 2016). In studies with cynomolgus monkeys with laser-induced CNV, dual inhibition of VEGF and ANG-2 with faricimab showed superior efficacy compared to just VEGF-A inhibition with ranibizumab (Regula *et al.,* 2016). Contrary to nesvacumab, it has successfully completed phase II trials and two phase III programmes with DME patients are anticipated to commence in 2019 (Roche, 2018). On the other hand, supplementing anti-VEGF therapy with the small-molecule pegpleranib, an inhibitor of another angiogenic protein, platelet-derived growth factor (PDGF) inhibitor, or with the anti-PDGF receptor β mAb rinucumab provided no benefit in wet AMD therapy, prompting researchers to re-evaluate PDGFs role in ocular angiogenesis (Chakravarthy *et al.,* 2017).

Tissue factor (TF) is a cell-surface receptor for plasma coagulation factor VII (fVII) and its activated form fVIIa, that trigger its functions as an activator of blood coagulation and in regulating both physiological and pathological angiogenesis (Wang *et al.,* 2014; Wells *et al.,* 2018). Whereas TF has not been detected in normal choroidal epithelium or ocular vasculature, the expression of TF is upregulated in the retinas of AMD patients, in which TF is considered a key contributor to the underlying inflammatory and angiogenic processes and to the overall progression of the disease (Hu, 2018; Wells *et al.,* 2018). Hence, different TF-targeting approaches are studied and evaluated, and several TF-targeting mAbs are in preclinical and clinical studies for treatment of AMD and other pathological neovascular diseases (Hu, 2018). For example, an anti-TF mAb decreased TF and VEGF expression, and reduced lesion area in a murine model of CNV (Wang *et al.,* 2014). An antibody-like Fc immunoconjugate ICON-1 has two proteolytically inactivated human fVIIa as targeting domains and blocks endogenous fVII by binding to TF with high affinity. Moreover, as the Fc domain acts as an effector, ICON-1 bound to TF on the surface of new vessels can also trigger the cytolytic reduction and/or removal of this pathological neovasculature. Single IVT ICON-1 injections were shown to be safe and

well-tolerated in a completed phase I/II trial also demonstrating preliminary evidence of efficacy against CNV in wet AMD patients (Wells *et al.,* 2018). This prompted progression to a phase IIa study in wet AMD patients where ICON-1 administered as monotherapy and in combination with ranibizumab was compared to ranibizumab monotherapy (Hu, 2018). At 6 months, ICON-1 showed signs of biological activity as patients receiving combination therapy had visual acuity gains comparable with ranibizumab but required fewer treatments and had longer treatment-free intervals (Gonzalez & Burian, 2017). Further efficacy studies are ongoing as repeated intravitreal ICON-1 injections both in combination with and as a maintenance therapy after aflibercept are evaluated in a phase IIb study in wet AMD patients with CNV (Iconic Therapeutics, Inc., 2018).

Regardless of the promise and achieved success of various anti-angiogenic biologicals, the several unfortunate failures cannot be overlooked but ought to be heeded as words of warning. Retinal physiology and pathological ocular angiogenesis involve the interplay of several pro- and anti-angiogenic factors (Cabral *et al.,* 2017). Hence, it is becoming clear that a more comprehensive understanding of their functions, cooperation and overall contribution in the maintenance of retinal vasculature and the pathogenesis of diseases must be attained before substantial breakthroughs, such as fully counteracting ocular pathologic angiogenesis, can be reached.

Immune modulation and complement inhibition

As the immune system is considered to play key roles in the pathology of, for example, AMD, targeting and modulating associated components, both innate and adaptive, are potentially viable as therapeutic strategies (Gehrs *et al.,* 2006; Hollanders *et al.,* 2015; Kim *et al.,* 2015; Volz & Pauly, 2015; Bandello *et al.,* 2017). Studies linking increased activation of the adaptive immune system to AMD development and progression have led to multiple trials evaluating the augmentation of existing anti-VEGF therapies with intravitreal and systemic anti-inflammatory biologicals (Volz & Pauly, 2015).

Elevated levels of cytokines, and especially certain interleukins (IL), such as IL-6, have been observed in DME patients and IL-6 is also associated with DR pathogenesis and breakdown of BRB (Agarwal *et al.,* 2015). The feasibility of IL-6 blockage as a DME treatment was confirmed with EBI-029, a humanized anti-IL-6 antibody, which was shown to reduce CNV in a preclinical rat model (Schmidt *et al.,* 2014). Furthermore, much like with teprotumumab, the pathophysiology of DR is exploited with the intravenous administration of humanized anti-IL-6 receptor mAb tocilizumab, a treatment approach currently in phase II trials in DME patients (Agarwal *et al.,* 2015; Stewart 2017). Additionally, combining systemic daclizumab, a humanized mAb targeting IL-2 receptor subunit α, with intravitreal anti-VEGF therapy decreased the number of required injections in a phase II study in AMD patients with CNV (Nussenblatt *et al.,* 2010). On the other hand, augmenting intravitreal anti-VEGF therapy with systemic infliximab, a chimeric mAb against the pro-inflammatory cytokine tumor necrosis factor α (TNF-α), failed to demonstrate additional benefit and did not reduce the number of required IVT injections in the same preliminary study. Some case reports have nonetheless described patients benefiting from intravitreally administered immunosuppressants, such as infliximab

and adalimumab (a humanized anti-TNF-α mAb) (Pascual-Camps *et al.,* 2014) and even from systemic adalimumab (Fernández-Vega *et al.,* 2016), raising cautious optimism of immunosuppressive (co-)therapy in AMD. While the roles of , e.g., TNF-α and other cytokines are recognized in the pathogenesis of retinal neovascularization and macular edema, definitive evidence of beneficial effects of immune mediators in AMD and other macular diseases remain somewhat sparse, calling for further preclinical and clinical assessment (Nussenblatt *et al.,* 2010; Pascual-Camps *et al.,* 2014; Volz & Pauly, 2015; Mandal *et al.,* 2018).

The complement system is a tightly controlled cascade of mediators that acts as part of the innate immune system in defense against, e.g., pathogens (Gehrs *et al.,* 2006). Dysregulation of the local complement system has been linked to AMD and especially to GA (Ferrington *et al.,* 2016; Holz *et al.,* 2018). Several biologicals targeting different factors of the complement system have been evaluated preclinically and in clinical trials for ocular indications (Volz & Pauly, 2015; Holz *et al.,* 2018). Lampalizumab is a humanized Fab fragment that selectively inhibits complement factor D but failed to demonstrate efficacy in treating GA in a phase III trial with dry AMD patients. As other clinical trials have proven other complement inhibitors, such as eculizumab and tesidolumab, ineffective as monotherapies against GA, this warrants questions on the feasibility of complement inhibition as a therapeutic strategy, at least in GA treatment (Dolgin, 2017; Holz *et al.,* 2018). Again, these clinical trial failures underline the need for a more comprehensive understanding of the pathophysiology of these complex diseases, especially when novel modalities are developed and evaluated.

Neuroprotection

The involvement of various cytokines, growth factors and neurotrophic factors has been implicated in posterior segment diseases and their application in patients with posterior segment disease has piqued researchers' interest for decades (LaVail *et al.,* 1992; Birch *et al.,* 2013). Furthermore, their ophthalmologic uses are not limited to just conditions with certain etiologies and they are therefore possibly more universally applicable (Pardue & Allen 2018). Different neuroprotective approaches range from utilizing exogenous proteins to targeting the receptors and signaling pathways of these proteins (Kimura *et al.,* 2016).

One of the most studied neurotrophic factors, the neuropoietic cytokine ciliary neurotrophic factor (CNTF), has *in vitro* been shown to protect rod and cone photoreceptors from neurodegeneration and light damage (Chong *et al.,* 1999; Sieving *et al.,* 2006; Li *et al.,* 2010; Li *et al.,* 2018), enhance axonal regeneration and RGC survival (Van Adel *et al.,* 2005; Leaver *et al.,* 2006; Cen *et al.,* 2007; Müller *et al.,* 2009), and increase RPE survival (Li *et al.,* 2011). Several *in vivo* studies with CNTF and Axokine (an engineered recombinant human CNTF analogue; Panayotatos *et al.,* 1993) have rather consistently demonstrated CNTF-mediated rescue of photoreceptors and/or improvement of their function in different animal models of retinal disease, such as glaucoma and optic nerve injury (Ji *et al.,* 2004; Leaver *et al.,* 2006; Pease *et al.,* 2009; Kimura *et al.,* 2016), diabetic retinopathy (Ma *et al.,* 2018), CNGB3-achromatopsia (Marangoni *et al.,* 2015), retinitis pigmentosa (Tao *et al.,* 2002; Li *et*

al., 2010; Rhee *et al.,* 2013; Lipinski *et al.,* 2015), and pathological neovascularization (Bucher *et al.,* 2016). CNTF's translation to the clinic has, however, been arduous.

In preclinical studies, CNTF has been delivered directly via IVT injection in most models, but this approach has been deemed infeasible for long-term treatment due to several limitations (Wen *et al.,* 2012). Like most neurotrophic factors, CNTF is an unstable protein (Hottinger & Aebischer, 1999; Fandl *et al.,* 2006), and has a short vitreal half-life with diffusion and protein degradation possibly accounting for the rapid clearance (Leon *et al.,* 2000; Beltran *et al.,* 2007). Therefore, as the effects of IVT administered CNTF are transient, frequent invasive IVT injections would be required for achieving sustained neuroprotection, a significant impediment as many posterior segment diseases can progress slowly over years and decades and making this approach impractical for long-term neuroprotection (Tao *et al.,* 2002; Ghasemi *et al.,* 2018). Moreover, the fact that exogenous CNTF can paradoxically suppress retinal function and impair vision in a dose-dependent manner (Wen *et al.,* 2006; Beltran *et al.,* 2007; McGill *et al.,* 2007) also prevents administering CNTF as large IVT bolus. This effect has been observed e.g. as suppressed electroretinogram responses and attributed to CNTF-induced biochemical and morphological changes in the phototransduction machinery of rod photoreceptors and was shown to be fully reversible (Wen *et al.,* 2006). Nevertheless, efficient means for avoiding associated adverse effects of repeated bolus injections are not available. Therefore, clinical trials with IVT administered CNTF injectables were not initiated (Tao *et al.,* 2002; Sieving *et al.,* 2006).

Various measures have been taken to overcome these limitations, most often by providing sustained CNTF release into the vitreous. NT-501 (a.k.a. Renexus®), an intraocular implant with immortalized CNTF expressing cells encapsulated inside a semi-permeable capsule, has been studied in several clinical studies in GA and RP (Ghasemi *et al.,* 2018). Despite successful proof-of-concept studies (Zhang *et al.,* 2011), Renexus® therapy eventually failed in GA and RP patients due to disappointing efficacy and clinical outcomes (Kauper *et al.,* 2012; Birch *et al.,* 2013; Birch *et al.,* 2016). Different reasons for these failures, including poor understanding of disease pathology and hence choice of therapeutic, have been discussed (Wong *et al.,* 2017). However, other indications for Renexus® are still actively explored. Following observations in earlier phase I trials, Renexus® is currently in phase II trials in glaucoma patients (Ghasemi *et al.,* 2018), and after encouraging efficacy results in a completed phase II trial in patients with macular telangiectasia type 2 (Chew *et al.,* 2019), patient recruitment for two parallel phase III trials with Renexus® is currently ongoing. These clinical trials are far from completion and comprehensive results pertaining efficacy are still a few years down the road. Based on dose-response studies, CNTF-mediated impairment of retinal function is unlikely to be of concern with therapeutic CNTF doses (McGill *et al.,* 2007) and therefore neither with the Renexus® approach. Some caution is nevertheless warranted and human patients receiving such CNTF-based treatment should be monitored closely until the clinical relevance and possible impact of CNTF-mediated negative regulation is conclusively resolved. Nevertheless, as the feasibility of the concept has been demonstrated – not only with CNTF, but also with soluble VEGFR-1 (Kontturi *et al.,* 2015) and a complement inhibitor (Annamalai *et al.,* 2018) – such encapsulated cell technologies (ECT) are an

altogether attractive approach to circumvent frequent IVT injections of biologicals in treating (vitreo-)retinal and choroidal diseases (Wong *et al.,* 2017).

IVT administered nerve growth factor (NGF) protects retinal cells from damage in an induced retinal detachment model in rats (El Sanharawi *et al.,* 2010). There are, however, conflicting reports of NGF's effects on RGC survival and regeneration. Interestingly, reduced progressive RGC loss and improved visual outcomes in hypertensive eyes of rats were observed upon topical NGF administration (Lambiase *et al.,* 2009). Although the same study evaluated the safety of topical NGF in a first-in-human trial with 3 glaucoma patients, and a different pilot study in RP patients reported of a subjective feeling of improved vision in 3 out of 8 patients receiving NGF eye drops (Falsini *et al.,* 2016) further studies are required to demonstrate the efficacy of NGF treatment, for example, in patients with retinal neurodegenerations (Kimura *et al.,* 2016).

The brain-derived neurotrophic factor (BDNF) is a potent neuroprotective agent. In the eye it is critical to RGCs in retinal development and in response to optic nerve injury, as well as in visual system function (Kimura *et al.,* 2016; Mysona *et al.,* 2017). Reduced lachrymal and optic disc BDNF levels have consistently been observed in glaucoma patients, making BDNF a potential glaucoma biomarker. Exogenous BDNF protects RGCs from damage, and the induction of BDNF overexpression by virally mediated gene therapy and other agents also promotes RGC survival (Ko *et al.,* 2001; Mysona *et al.,* 2017), whereas it can also slow down photoreceptor degeneration in mice (LaVail *et al.,* 1998). As with NGF, topical BDNF eye drops are effective in rescuing visual function in mice, although both strategies remain to be validated in humans (Domenici *et al.,* 2014; Mysona *et al.,* 2017). Even so, BDNFs effects on neuroprotection and in delaying RGC are short-lived and even repeated IVT injections cannot guarantee RGC survival, making its therapeutic value in posterior segment diseases limited especially when used alone (Klöckler *et al.,* 1998; Ko *et al.,* 2001; Harada *et al.,* 2015). Furthermore, regardless of the positive effects, the ocular application of BDNF has been shown to upregulate nitric oxide synthase, which in turn is harmful to RGCs (Klöckler *et al.,* 1998; Zhang *et al.,* 2005).

Glial cell-line-derived neurotrophic factor (GDNF) exerts neuroprotection on retinal cells in different retinal pathologies, and for example RGC rescue as well as retardation of photoreceptor degeneration have been demonstrated along with functional rescue (Hauck *et al.,* 2006). This neuroprotection takes place even at low GDNF concentrations provided that its delivery is maintained long-term (García-Caballero *et al.,* 2017). Interestingly, GDNF is observed to exert its neurotrophic effects on photoreceptors both in a direct and an indirect manner; in mouse and porcine retinas, receptors for GDNF have been found mostly in retinal Müller glial cells instead of photoreceptors themselves (Delyfer *et al.,* 2005; Hauck *et al.,* 2006). These cells in turn mediate the upregulation and release of other factors shown to support photoreceptors directly, a mechanism proposed also for other neurotrophic factors (Hauck *et al.,* 2006; Harada *et al.,* 2015; Kimura *et al.,* 2016). All things considered, GDNF has been shown to increase RGC survival in preclinical animal models of glaucoma and as such it displays therapeutic potential in the treatment of glaucoma and RP.

Mesenchephalic astrocyte-derived neurotrophic factor (MANF) was recently shown to be expressed in the mouse retina, where it promotes tissue repair and

increases successful regeneration (Neves *et al.,* 2016). RGCs were subsequently shown to express MANF and to respond to exogenous MANF treatment in *in vitro* and *in vivo* models of glaucoma and RP, concluding MANF to be a potential therapeutic candidate for promoting RGC survival (Gao *et al.,* 2017; Lu *et al.,* 2018).

Whereas several neurotrophic factors have untapped therapeutic potential in neurodegenerative diseases of the CNS and posterior eye segment, their pharmaceutical development is by-and-large still in the earlier stages with CNTF and NGF as the only one to have been tested in the eye in clinical trials (Kimura *et al.,* 2016; Pardue & Allen 2018). Furthermore, as their effects are tightly linked to dosage and as high IVT doses may in fact be harmful to retinal function, the continuous delivery of small doses is likely safer and more effective (El Sanharawi *et al.,* 2010).

Combating oxidative stress and toxic byproduct buildup

The presence of misfolded and aggregated proteins is associated with AMD. It has been suggested that this arises partly due to age-related chronic oxidative stress and overwhelmed clearance capacity (Kivinen *et al.,* 2014; Kaarniranta *et al.,* 2017). Hence, the prevention and removal of protein aggregates has been proposed as a means of controlling and/or hindering the progress of this disease (Kivinen *et al.,* 2014; Ferrington *et al.,* 2016). Heat shock proteins (Hsps) function as molecular chaperones, i.e., in maintaining and restoring correct 3D structures of nascent and misfolded proteins, and in preventing the formation and accumulation of potentially cytotoxic protein aggregates in cells (Yu *et al.,* 2001). Should this fail, proteins are targeted for proteasomal disposal (Kivinen *et al.,* 2014). Furthermore, Hsps inhibit apoptosis, a mechanism of programmed cell death that occurs also in many posterior segment diseases (O'Reilly *et al.,* 2010). Consequently, elevated retinal levels of Hsps – induced, e.g., in response to oxidative stress – have been observed in AMD patients (Decanini *et al.,* 2007; Urbak & Vorum 2010; Ferrington *et al.,* 2016). Interestingly, it also seems that such a capability of mounting a response to stress declines in aging cells, such as in the RPE of AMD patients (Kivinen *et al.,* 2014; Subrizi *et al.,* 2015). Therefore, strategies utilizing the cytoprotective activities of Hsps in neuroprotection of the retina has generated interest (O'Reilly *et al.,* 2010).

The widely studied Hsp27 and Hsp70 proteins are strongly induced by stress, and as they play important roles in protecting the retina, they are attractive as potential therapeutics in retinal diseases (Urbak & Vorum 2010; Chidlow *et al.,* 2014). Hsp27 has shown protective effects on neuronal cells against apoptosis and is a therapeutic candidate, for example, for protecting RGCs in glaucoma (Kalesnykas *et al.,* 2007; O'Reilly *et al.,* 2010). Potential of Hsp70 in photoreceptor survival after retinal detachment – a condition associated with various retinal diseases – was noted as Hsp70 was up-regulated in an *in vivo* animal disease model and prevented the activation of cell death pathways, whereas administration of a Hsp70 inducer was also demonstrated to result in decreased photoreceptor apoptosis (Kayama *et al.,* 2011). *In vitro*, Hsp70 was shown to bind to perinuclear protein aggregates under proteasome inhibition in ARPE-19 cells; owing to decreased lysosomal enzyme activity the proteasomal degradation diminishes during aging in the RPE, resulting in lipofuscin and protein aggregate formation and accumulation (Kivinen *et al.,* 2014). Hsp70 has also been found localized in the lysosomes of the RPE cells, where its functions may

involve the maintenance of the lysosomal enzymes' activity in the removal of harmful protein species. Furthermore, after uptake into ARPE-19 cells, exogenous rhHsp70 localized in late endosomes and lysosomes and protected the cells from oxidative stress, while diffusion of IVT rhHsp70 from vitreous to the RPE was observed in a porcine *ex vivo* model, providing a proof-of-concept of targeting lysosomal protein aggregates with a Hsp70 chaperone as a potential AMD therapy (Subrizi *et al.,* 2015). The neuroprotective therapeutic utility of Hsp70, especially in acute retinal traumas, is also appealing as IVT administered Hsp70 reduced photoreceptor cell apoptosis after light damage in rat eyes *in vivo* (Yu *et al.,* 2001). Despite numerous studies providing proof of the utility of Hsp therapy in posterior segment diseases, sustained delivery approaches for long-term neuroprotection with Hsps seem to be lacking, and there are notable gaps in our understanding of the roles of Hsps in the eye and pathology of ocular diseases (Urbak & Vorum 2010; Chidlow *et al.,* 2014). Hence, regardless of their considerable promises, treating ocular diseases with Hsp therapeutics is still far from realisation in the clinic.

Amyloid beta (Aβ) is a family of highly toxic, aggregation-prone peptides most often associated with neurodegenerative disorders, e.g., Alzheimer's and Parkinson's (Ratnayaka *et al.,* 2015). The presence of elevated levels of Aβs in the ageing retina, and especially in the extracellular debris deposits known as drusen, has been linked to AMD pathogenesis and progression, and was shown to lead to downregulation of PEDF and upregulation of VEGF expression in *in vitro* human RPE cultures (Yoshida *et al.,* 2005; Ratnayaka *et al.,* 2015; Radhakrishnan *et al.,* 2017). Two Aβ-targeting antibodies have been developed to attenuate such effects via preventing the accumulation of Aβs and thus reducing the pool of these toxic compounds; an intravenously administered mAb and a different intravitreally administered mAb have both undergone phase II studies in patients with dry AMD (Singer 2014; Radhakrishnan *et al.,* 2017).

2.4.3 Drug delivery strategies for biologicals

Although novel biologicals are being developed for ocular indications, the innate obstacles of their use in ocular pharmacotherapy remain (Mandal *et al.,* 2018). As a result, routine administration still takes place by IVT injections in the clinic (Radhakrishnan *et al.,* 2017). To improve the efficacy and ocular PK of both approved and emerging biologicals, numerous research efforts exploring different drug delivery approaches are ongoing (El Sanharawi *et al.,* 2010; Agrahari *et al.,* 2017; Radhakrishnan *et al.,* 2017; Lau *et al.,* 2018).

To mitigate the safety risks and treatment burden associated with frequent IVT injections, one of the main development goals is to achieve less frequent and less invasive dosing, i.e., to make treatment more amenable to patients and clinicians alike. As with delivery of biologicals in general, strategies to improve ocular delivery also range widely (Mitragotri *et al.,* 2014; Patel *et al.,* 2014). From therapeutic protein engineering approaches to implantable systems and nanocarrier-based drug delivery systems, the approaches are far too numerous to be included here, and have been comprehensively reviewed elsewhere (Shah *et al.,* 2010; Thrimawithana *et al.,* 2011; Patel *et al.,* 2013; Radhakrishnan *et al.,* 2017; Lau *et al.,* 2018). Instead, chosen examples are presented in the Appendix as **Table S2** where some of the more studied

and established approaches and strategies are briefly discussed, and the reader is kindly directed to a recent paper by Mandal *et al.,* (2018) for a more in-depth review. Although different physical forces/phenomena, such as electrical fields and sonophoresis, and their application to transiently disrupt biological barriers and enhance the ocular penetration of biologicals are actively studied (Huang *et al.,* 2018), for brevity, they are not further discussed here.

Therapeutic protein engineering approaches

As discussed in sections 2.2.2 and 2.3.1, retaining therapeutic protein stability is of paramount importance in ensuring treatment efficacy and safety. Although models to evaluate therapeutic protein stability in the vitreous have been developed (Patel *et al.,* 2015; Patel *et al.,* 2017), they cannot fully represent the *in vivo* situation whereas studying stability in a patient-setting is impractical and unethical. Among other challenges, sustained ocular delivery strategies may necessitate very high protein concentrations whereas the protein itself needs to be resistant to different stresses and extended exposure to varying conditions (Tesar *et al.,* 2017). By utilizing protein engineering to modify the protein, a Fab fragment for formulation in high protein concentrations and for enhanced stability in vitreal conditions was developed with the aim of enabling less-frequent dosing (Tesar *et al.,* 2017). Whereas improved stability under both formulation and physiological were achieved with this approach, follow-up studies in humans are needed to corroborate these findings.

Many ocular drug delivery strategies are based on exploiting the eye's physiology. The fusion of anti-VEGF mAbs together with a hyaluronic acid (HA) binding peptide increased the intraocular half-lives 3–4 fold compared to unmodified proteins *in vivo* (Ghosh *et al.,* 2017). As a major macromolecular component of the vitreous, HA in this case acts as an intraocular reservoir for bound protein, slowing the clearance of the mAb and thus extending their therapeutic efficacy. Likewise, the binding of a Nanobody® to intravitreal albumin was shown to result in a 3-fold increase in its vitreal half-life (Fuchs & Igney, 2017), although this was most likely due to an increase in hydrodynamic size that results in decreased anterior clearance (Mehta *et al.,* 2019). While this strategy might allow for prolonged dosing intervals, intravitreally administered albumin-binding biologicals may have their drawbacks. In addition to the limited binding capacity of vitreal albumin, the half-life extension imparted by albumin-binding may not be sufficient, as albumin itself has an intravitreal half-life of only a few days, whereas after ocular clearance the systemic half-life of albumin-binding biologicals may be unacceptably long (Ghosh *et al.,* 2017; Mehta *et al.,* 2019).

Aside from specific binding to vitreal components, increasing residence time by modulating molecular size and/or hydrodynamic radius seems to be a viable strategy as increasing evidence points it to be a critical factor in intravitreal clearance of biologicals (del Amo & Urtti, 2015; Shatz *et al.,* 2016; Hutton-Smith *et al.,* 2017; Shatz *et al.,* 2019). For example, the hydrodynamic radii of abicipar and pegaptanib are increased through PEGylation, which prolongs their residence times (Shatz *et al.,* 2018). PASylation®, i.e., conjugating/fusing polypeptides composed of Pro, Ala and/or Ser, is a technique complementary to PEGylation that has been utilized to extend ocular residence of an anti-VEGF Fab fragment (Gebauer & Skerra, 2018).

Other approaches include utilizing covalent grafting of multiple Fab fragments and VEGF decoy receptors to HA (Altiok *et al.,* 2016; Famili *et al.,* 2019), and branched PEG (Shatz *et al.,* 2019) to prolong the vitreal residence time.

FcRn binding is routinely exploited with Fc-fusion proteins, such as aflibercept and etanercept, and is also possible with albumin-fusion proteins (Sockolosky & Szoka, 2015). Further modulation of this interaction to influence the PK of therapeutic proteins is also possible. For example, whereas bevacizumab is recycled and salvaged by FcRn, the Fc-fusion protein aflibercept was present in FcRn-mediated recycling-associated organelles but also observed to be destined for lysosomal degradation in a bovine *in vitro* model (Deissler *et al.,* 2018). Hence, further engineering of aflibercept could be of utility to influence this interaction and direct the protein more efficiently to be recycled and salvaged. On the other hand, as FcRn-mediated transport over the BRB contributes to the ocular elimination of bevacizumab (Kim *et al.,* 2009), avoiding the FcRn binding of biologicals could instead be preferable in ocular use (Shah, 2015). One can argue that this would in turn also abolish FcRn-mediated recycling of endocytosed proteins, possibly resulting in overall shortened vitreal half-life. There is also evidence pointing to the contrary (Gadkar *et al.,* 2015) although it should be noted that the overall relevance of posterior clearance mechanisms is – albeit under debate as quantitative data elucidating this is lacking – considered small (5–15%) compared to anterior clearance (del Amo *et al.,* 2017; Deissler *et al.,* 2017; Shatz *et al.,* 2018; Mehta *et al.,* 2019; Ramsay *et al.,* 2019). Nonetheless, different factors influence the FcRn-binding of biologicals and serve as clues for protein engineering approaches (Oganesyan *et al.,* 2014). Accordingly, multiple different strategies to modulate this interaction exist, and although they have mostly been utilized to impact systemic PK (Roopenian & Akilesh, 2007; Sockolosky & Szoka, 2015), such modulation could also be explored in ophthalmologic biologicals. For example, to influence the pH-dependent binding, the affinity of the interaction can be altered via engineering the Fc domain by, e.g., mutating amino acids responsible for the interaction (Borrok *et al.,* 2015; Sockolosky & Szoka, 2015). Moreover, as regions distal to the Fc domain have been observed to interact with FcRn-binding (Schoch *et al.,* 2015; Piche-Nicholas *et al.,* 2018), engineering these parts could also be carried out to tune the ocular PK of biologicals. All in all, the significance of FcRn-binding in the ocular PK of biologicals is still not fully established. The overall relevance of all posterior clearance is considered small, and strategies to modulate the FcRn interaction may therefore have minimal impact on the intravitreal half-lives of biologicals, although this cannot yet be comprehensively stated.

Drug delivery systems and implants

To address and mitigate the challenges of frequent IVT injections, sustained and controlled release formulations, such as liposomes, hydrogels, micro- and nanoparticles, and different polymer matrix systems have been developed for biologicals (Agrahari et al., 2017; Joseph & Venkatraman, 2017; Iyer et al., 2019). Furthermore, there is also great interest in the development of stimuli-responsive drug delivery systems, capable of releasing biologicals upon external stimulus, such as light or ultrasound (Agrahari et al., 2017).

A recent study utilizing an *ex vivo* set-up to mimic sub-conjunctival administration and trans-scleral transport demonstrated controlled release of liposome-encapsulated ranibizumab (Joseph et al., 2017); the study, however, only quantitated trans-scleral transport whereas ranibizumab's activity after said transport was not determined. Likewise, GDNF co-encapsulated with vitamin E was released from biodegradable poly-lactic-co-glycolic acid (PLGA) microspheres after a single IVT injection to the rabbit vitreous for up to 6 months in a controlled fashion resulting in concentrations that were earlier demonstrated to protect RGCs in an animal glaucoma model (García-Caballero et al., 2017). Similarly, sustained release of aflibercept from polymeric PLGA nanoparticles over 7 days has been described *in vitro*, although retention of aflibercept's therapeutic efficacy in such a system remains to be demonstrated *in vivo* (Kelly *et al.,* 2018). Many of these developments have, however, not been without considerable hurdles. Although the dual dAb protein retained activity for over 12 months and showed protection over 6 months in a primate model of wet AMD upon sustained release from microparticles, particle migration and adverse effects were also described (Adamson *et al.,* 2016). Whereas each approach has advantages and challenges, common obstacles pertaining to (nano)carriers include the issues of reproducibility, scalability, complex fabrication and long-term stability in biological fluids. These problems must be solved prior to successful clinical translation (Agrahari *et al.,* 2017; Joseph & Venkatraman, 2017).

Implantable devices are another approach for sustained drug delivery and prolonged dosing intervals (Shah *et al.,* 2010; Shatz *et al.,* 2018). The Port Delivery System is a surgically implanted, non-biodegradable and refillable drug reservoir on the sclera that allows for the continuous release of therapeutic concentrations of biologicals into the vitreous over extended periods (Lau *et al.,* 2018). It has been developed to reduce the need for repeated injections and as a less invasive administration method it reduces the burden of injections. The Port Delivery System was evaluated in a recent phase II clinical trial for the extended delivery of ranibizumab in wet AMD patients and is currently recruiting for pivotal phase III studies (Campochiaro *et al.,* 2019). Similarly, the cell-based implant system Renexus® discussed in section 'Neuroprotection' on page 38 is an implantable bioreactor for the sustained *in oculo* production of CNTF over extended time periods, and ECT systems have been developed for the sustained intravitreal release of other ophthalmologically relevant biologicals (Kontturi *et al.,* 2015; Annamalai *et al.,* 2018). Lastly, different extraocular reservoir systems have been designed for convenient refilling and sustained release (Joseph & Venkatraman, 2017; Lau *et al.,* 2018). The MicroPump System – a technology akin to insulin pumps – is a pump system that delivers biologicals in a controlled fashion and has been evaluated in a small trial for controlled release of ranibizumab. Even though the system was well tolerated, there were significant challenges with the implementation of this system and suboptimal drug effects in the study. It is unclear whether the developers will proceed further with the system (Lau *et al.,* 2018; Shatz *et al.,* 2018).

Although various drug delivery systems have been developed to provide sustained and less invasive solutions for the ocular delivery of biologicals, translation to clinical applications has been challenging. For example, material safety and toxicity, carrier properties, loading capacities, maintaining therapeutic protein integrity and activity, as well as differences in posterior segment diseases are but some

of the many roadblocks that have hampered scientists from reaching this goal. Even with active research and rapid progress in the field, no system has advanced to the clinics (Shah *et al.,* 2010; Delplace *et al.,* 2015; Agrahari *et al.,* 2017; Radhakrishan *et al.,* 2017). Hence, research on retinal disease pathologies, their models, as well as determinants of ocular barriers and PK of biologicals is still needed. Accordingly, close collaboration across scientific areas and fields is needed to bring innovative ocular drug delivery systems into the clinic to rescue and even restore vision in patients.

2.5 Other approaches

As the underlying pathogenesis of retinal diseases are diverse, in addition to the biologicals discussed in previous sections, various other treatment strategies have been explored. For example, in response to inflammation implicated in the pathogenesis of many posterior segment diseases (Pascual-Camps *et al.,* 2014), several anti-inflammatory strategies have been explored and developed, including the intraocular implants Iluvien® and Ozurdex® for the sustained intravitreal release of fluocinolone acetate and dexamethasone for treating DME (Wang & Lo, 2018). Moreover, small molecules inhibiting the downstream signaling of VEGFRs have been explored as strategies against pathological neovascularization (Delplace *et al.,* 2015; Volz & Pauly 2015). Apart from small molecule drugs, biologicals, and drug delivery strategies and systems to enhance and prolong their ocular delivery, other forms of therapy for retinal diseases are also under development. For brevity, only gene therapy and cell-based therapies are briefly discussed hereafter, and the reader is kindly pointed towards recent reviews on the subjects for more thorough summaries (Agrahari *et al.,* 2017; Aguirre, 2017).

Genetic predispositions contribute to many posterior segment pathologies (Aguirre, 2017). Gene therapy offers considerable hope in combating certain hereditary posterior segment diseases and is also of interest for achieving prolonged release of therapeutic proteins in general (El Sanharawi *et al.,* 2010; Agarwal *et al.,* 2015). In other words, gene therapy may not only be utilized to replace a mutated gene or to supply a missing protein, but also to replace repeated injections. Furthermore, as an individual patient's genetic makeup may influence the choice of therapy, treating AMD, for example, may become more personalized in the future (Yang *et al.,* 2016). Thus far, retinal gene delivery with intravitreally injected non-viral and viral vectors have been studied in preclinical and clinical trials (Aguirre*,* 2017; Awwad *et al.,* 2017). Adenovirus and the adeno-associated virus have most often been utilized in the delivery of, for example, CNTF and PEDF gene therapies (Ishikawa *et al.,* 2015; Lipinski *et al.,* 2015; Ghasemi *et al.,* 2018). To give a more detailed example, an adenovirus vector carrying a gene for sFLT-1 (soluble VEGFR-1) has been developed to induce the cells of the outer retina to express and secrete soluble anti-VEGF receptor that in turn binds VEGF, inhibiting it from binding to its receptors in the retina (Stewart, 2018b). Although proof-of-concept was acquired with this treatment in wet AMD patients, development was halted due to lower than expected performance. Whereas the potential in treating retinal pathologies is considerable, direct cellular delivery and intracellular release of the therapeutic gene are but some of the many challenges and obstacles of gene therapy (Awwad *et al.,* 2017). Regardless of major

hurdles and setbacks, the first ever retinal gene therapy was approved by FDA in 2018 (Ameri, 2018). As gene editing has advanced considerably with new tools, such as CRISPR/Cas9 becoming available, other gene therapy approaches for retinal pathologies can be envisioned to follow suit.

The possibilities of regenerative therapies, such as utilizing cell transplants in replacing degenerated photoreceptors and RPE are considerable as such transplants could potentially not only preserve vision but even restore lost vision (Delplace *et al.,* 2015; Zarbin, 2016). Aside from direct replacement, cells engineered to excrete, for example, CNTF and GDNF could also be transplanted to achieve long-term *in situ* release of these therapeutic, neuroprotective proteins (Awwad *et al.,* 2017; Oswald & Baranov, 2018). Several early stage clinical trials are currently ongoing – for example in GA patients – to evaluate the safety and efficacy of cell therapies derived from both human embryonic stem cell cells and induced pluripotent stem cells (Zarbin, 2016; Oswald & Baranov, 2018). Regardless of notable fundamental obstacles that have been addressed, significant challenges such as cell manufacture, delivery and survival remain to be overcome before cell-based therapies can be implemented as routine clinical treatment alternatives in retinal pathologies.

In conclusion, regardless of notable advances in the treatment of posterior eye segment diseases during the last 20 years, many of the obstacles remain to be tackled. Whereas management of diseases such as wet AMD and DR has progressed with the introduction of several biological anti-VEGF therapies, some patients are resistant to these treatments that, regardless of all efforts, still require frequent injections to achieve clinical benefits. Many aspects of ocular PK of biologicals remain poorly understood and their significance has not been fully addressed. Even with emerging therapeutic modalities and drug delivery approaches, we are still learning, and it may become apparent that multiple pharmaceuticals and even multiple drug delivery systems are required for the successful treatment of certain conditions. Moreover, even though (therapeutic) protein production methods and tools have evolved and matured over the last decades, obtaining adequate protein yields still requires considerable effort and is at times a trial-and-error process. Likewise, new methods in therapeutic protein screening and development are in high demand to address and tackle the unsustainable trend of fewer successful biologicals entering the market in relation to expenditure.

3 Aims of the study

This thesis deals with the production and development of biologicals and their therapeutic applications in treating diseases of the posterior eye segment. The main focus of the investigations was on the ophthalmologically relevant protein, ciliary neurotrophic factor (CNTF).

The specific aims of this thesis were:

1. To screen for optimal conditions for the expression of soluble rhCNTF in *E. coli* (I)
2. To scale-up rhCNTF production and verify the functionality of rhCNTF (I)
3. To further characterize the purified rhCNTF (II)
4. To screen for appropriate storage formulations for purified rhCNTF and study the protein's long-term stability in those formulations (II)
5. To study the retinal permeation of rhCNTF (II)
6. To provide proof-of-concept for a novel production platform for streamlined pharmaceutical protein development (III)
7. To utilize the developed platform for bioconjugation of expressed target protein (III)

4 Materials and Methods

The methods and equipment employed in the studies are summarized in **Table 4**. Whereas well established and routinely used methods are not discussed here, key methods are discussed in more detail in the following sections. All materials and methods used in this thesis are described in full detail in the respective publications.

Table 4. *Overview of materials and methods used in the thesis work.*

Theme	Aim/Purpose	Objective	Methods, analytics	Publication
Expression vector construction	Generation of DNA fragments and assembly of plasmid vectors	Codon optimization for expression in *E. coli*	OptimumGene™ (Genscript, USA)	I
		Linearization of plasmid vectors	Restriction enzyme digestion, inverse PCR	I, III
		Amplification of gene inserts	PCR, gradient PCR	I, III
		Purification of DNA fragments and plasmid DNA	Gel and PCR clean-up, Mini-prep, Midi-prep	I, III
		Assembly of expression plasmids	Gibson Assembly™, NEBuilder®	I, III
		Validation of target gene insertion	Colony PCR, gene sequencing	I, III
rhCNTF production	Soluble rhCNTF expression	Screening of factors for optimal expression conditions in small scale	Small-scale IMAC with magnetic Ni^{2+}-beads, SDS-PAGE	I
		Estimation of soluble fraction of total rhCNTF expressed	SDS-PAGE	I
	Protein purification	Affinity purification	Batch IMAC with Ni^{2+}-resin, SDS-PAGE	I, II
		Removal of high molecular weight species	SEC, SDS-PAGE	I, II
Protein characterization	Protein detection	Protein identity, molecular weight, fragmentation, purity	SDS-PAGE, WB	I, III
	rhCNTF activity & function	Binding with cognate receptor subunit	rhCNTF biotin labeling, rhCNTF – rhCNTFRα-binding with ELISA	I
		rhCNTF-stimulated proliferation of TF-1.CN5a.1 cells	BrdU incorporation, ELISA	II
	rhCNTF folding and structure	Investigating secondary structure	CD	II
	rhCNTF conformational stability	Thermal unfolding of rhCNTF; observing T_h changes during storage	CD, ThermoFluor	II
	rhCNTF hydrodynamic size	Observing r_h changes during storage	DLS	II
	rhCNTF aggregation	Detection of high molecular weight species; aggregation upon heating	DLS	II

Table 4 continued.

Retinal PK of rhCNTF	Retinal permeation of rhCNTF	Distribution of rhCNTF in bovine and rat *ex vivo* retinal explants	Preparation of retinal explants, fluorescent labeling of rhCNTF, fluorescence microscopy	II
	Retinal localization of rhCNTF	Distribution of rhCNTF and microglia in rat *ex vivo* retinal explants	Preparation of retinal explants, fluorescent labeling of rhCNTF, Iba-1 staining, fluorescence microscopy	II
Accelerated protein development	Target protein expression	CFPS of rhCNTF-*Npu*DnaE$_{C\Delta16}$ in Tobacco BY-2 and HeLa lysates	WB	III
	Photocleavage of capture peptides	Photocleavage efficiency and rate of *Npu*DnaE$_{C16}$ peptides	UV_{365nm} exposure, HPLC	III
	Streamlined purification of protein of interest	Capture of PTS-generated *de novo* rhCNTF-His$_6$; light-induced release of rhCNTF	Ni^{2+}-capture of rhCNTF-His$_6$ generated via PTS, UV_{365nm} release of rhCNTF, WB	III
		Split intein-mediated capture of rhCNTF- *Npu*DnaE$_{C\Delta16}$ by Ni-NTA immobilized *Npu*DnaE$_{C16}$ peptide; light-induced release of rhCNTF	Split intein-mediated capture and UV_{365nm} release of rhCNTF with magnetic Ni^{2+}-beads, WB	III
Bioconjugation of rhCNTF	Biotinylation of rhCNTF	Split intein-mediated transfer of biotin from capture peptide to rhCNTF	Streptavidin-shift assay, WB	III

BrdU, 5-bromo-2'-deoxyuridine; CD, Circular dichroism spectroscopy; CFPS, cell-free protein synthesis; CNTF, ciliary neurotrophic factor; CNTFRα, CNTF α-receptor; DLS, dynamic light scattering; ELISA, enzyme-linked immunosorbent assay; HPLC, High-performance liquid chromatography; Iba-1, ionized calcium-binding adapter molecule 1, a microglial marker; IMAC, immobilized metal-ion affinity chromatography; Ni-NTA, nickel nitrilotriacetic acid; *Npu*DnaE, *Nostoc punctiforme* DnaE split intein; PCR, polymerase chain reaction; PK, pharmacokinetics; PTS, protein *trans* splicing; r_h, hydrodynamic radius; SDS-PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; SEC, size-exclusion chromatography; T_h, temperature of hydrophobic exposure; WB, Western blot

4.1 Binding assay with rhCNTFRα (publication I)

To indirectly assess the function of produced rhCNTF, an enzyme-linked immunosorbent assay (ELISA) assessing binding with the cognate α receptor was carried out. First, purified rhCNTF was reacted with 20:1 molar excess of N-hydroxysuccinimide (NHS)-PEG$_4$-biotin in a carbonate buffer solution in dimethylformamide, conjugating the biotin to exposed amines e.g. in the N-terminus and lysine side chains. After reaction, excess NHS-PEG$_4$-biotin was removed and the biotinylated rhCNTF (bCNTF) buffer exchanged to a phosphate buffer.

After coating the wells of a high protein-binding 96-well plate with rhCNTFRα, the wells were blocked with bovine serum albumin (BSA) to reduce non-specific binding to the plate surface. bCNTF was added to the wells and incubated to allow for binding to the rhCNTFRα, followed by washing the wells repeatedly to remove unbound protein. To detect the bound bCNTF, horseradish peroxidase (HRP)-conjugated streptavidin was added to the wells and observed colorimetrically by measuring the optical density of the well solutions after incubation with 3,3',5,5'-tertramethylbenzidine (TMB) substrate.

4.2 *In vitro* bioactivity study (publication II)

An *in vitro* cell study was carried out to verify that purified rhCNTF was active and able to trigger biological responses. CNTFRα expressing TF-1.CN5a.1 cells used in the experiments were maintained in a fully humidified 5% CO_2 atmosphere at 37°C as suspension cultures in a modified RPMI 1640 growth medium supplemented with fetal bovine serum (FBS), human granulocyte macrophage colony-stimulating factor (hGM-CSF), G-418, and penicillin/streptomycin.

The rhCNTF-mediated effects on TF-1.CN5a.1 cell proliferation was studied by measuring the incorporation of nucleotide analogue 5-bromo-2'-deoxyuridine (BrdU) during cell proliferation (Porstmann *et al.,* 1985). First, serial dilutions of purified rhCNTF were prepared in assay growth medium (complete growth medium without hGM-CSF and G-418) and added to wells in 96-well microplates for suspension cells. Supplements were removed from cultured TF-1.CN5a.1 cells by centrifugation and washing, and after resuspension in assay growth medium the cells seeded at 1.0 x 10^4 cells per well, yielding final rhCNTF concentrations ranging from 24 fg/ml to 100 ng/ml. Cells cultured in assay growth medium with and without hGM-CSF were used as positive and negative controls, respectively. rhCNTF and control treatments were carried out for 48 h.

After treatment, BrdU labeling solution was added and the cells were incubated to allow for incorporation of BrdU into the synthesized DNA during cell proliferation. After labeling, the cells were pelleted by centrifugation, the media aspirated from the wells, and the cells dried at 60°C, after which the cells were fixed and the DNA denatured with FixDenat solution. Followed by the removal of FixDenat solution, now allowing access to the incorporated and exposed BrdU, incubation with an HRP-conjugated anti-BrdU antibody was carried out. After antibody solution removal and repeated washing, TMB substrate solution was added to the wells, resulting in a colorimetric change that was subsequently quantitated by measuring the well absorbances.

4.3. Permeation of rhCNTF in ex vivo retinal explants (publication II)

To investigate whether rhCNTF can permeate into the retina, experiments mimicking *in vivo* ocular drug administration were carried out with *ex vivo* organotypic retinal explants prepared from bovine and rat eyes.

4.3.1 Fluorescent labeling of rhCNTF

For rat retinal explant experiments, purified rhCNTF was fluorescently labeled with Alexa Fluor™ 488 via tetrafluorophenyl ester linkage, conjugating the fluorophores to available amines, such as the N-terminus and exposed lysine side chains. Unreacted dye was separated from labeled protein with spin filters and the degree of labeling analyzed spectrophotometrically. Labeled rhCNTF was stored on ice until use.

For bovine retinal explant experiments, rhCNTF was fluorescently labeled with NT-647 via NHS ester linkage, likewise, to exposed amines. Unreacted dye was removed with spin columns and the degree of labeling analyzed spectrophotometrically. Labeled rhCNTF was stored in -80°C until use.

4.3.2 Retinal explant culture preparation and rhCNTF treatment

CD® (SD) IGS rat eyes were used to prepare *ex vivo* organotypic retinal explant cultures as described earlier (Arango-Gonzalez 2010, Caffe 2002). Young, 5, 6, or 8 days old animals were euthanized, and the eyes enucleated aseptically. After cleansing with ethanol, the eyes were briefly incubated in basal R16 medium followed by incubation in 0.12 % proteinase K for 15 min at 37°C to facilitate dissecting. To inactivate proteinase K the eyes were subsequently incubated in basal R16 medium containing FBS, and finally washed in serum-free basal R16 medium.

Dissections were carried out aseptically in a Petri dish with the eyes submerged in basal R16 medium. The anterior segment, sclera, choroid, lens and the vitreous body were delicately removed, leaving the retina with the attached RPE. Relaxing cuts were made and the retinae flat-mounted with the photoreceptor-side down on the porous culture membrane inserts i.e. at the air-medium interface. The cultures were maintained with complete R16 medium placed in the lower compartments of 6-well culture dishes at 36.5°C and 5% CO_2. No antibiotics or antimycotics were used.

Explants were left without treatment for 24 h to allow adaptation to culture conditions, followed by rhCNTF treatments for 24 h. Alexa Fluor™ 488-labeled rhCNTF was prepared in phosphate buffered saline (PBS), diluted in basal R16 medium and sterilized with membrane filter before use. Treatments were either applied apically directly as drops on the explant cultures, or basolaterally in complete medium in the lower compartment to mimic dosing via IVT and e.g. subretinal injections, respectively. Complete R16 medium was used as control.

Bovine retinal explant cultures were prepared as described earlier (Peynshaert *et al.,* 2017). First, fresh bovine eyes were cleaned of periocular tissues and disinfected with immersion in ethanol. The eye was bisected below the limbus, the anterior segment and the vitreous removed, with the remaining posterior eye cup filled with CO_2 independent medium and cut into 4 flaps. While submerged in medium, circular pieces of the retina were punched with a trephine blade and then transferred onto moisturized porous culture membrane inserts with the photoreceptor-side down. Explant culture medium supplemented e.g. with penicillin-streptomycin was added below the inserts and explant cultures maintained at 37°C and 5% CO_2. Treatments were given directly after explant preparation, with NT-647-labeled rhCNTF applied as drops on top of explants, followed by incubation for 24 h.

4.3.3 Tissue culture fixation and sectioning

Treated rat explants were fixed in 4% paraformaldehyde (PFA). After a short incubation at 4°C, the fixative was changed to 1% PFA and incubated further at 4°C overnight. Retinae were then washed with PBS and cryoprotected by incubation in graded sucrose solutions. Subsequently, tissues were embedded, with the vertical sections obtained on a microtome first air dried at 37°C, and then stored frozen until use.

Treated bovine explants were fixed by replacing the medium below the filter with 4 % PFA. After incubation at 4°C PFA was discarded and replaced with sucrose solution and incubated overnight at 4°C. Explants were snap-frozen in Tissue-Tek® O.C.T Compound using liquid nitrogen and sections cut from four different region of the explant with a microtome-cryostat.

4.3.4 Culture staining and imaging

To prepare tissue sections from rat retinal explants for imaging, frozen cryosections were first air-dried. Slides with fixed sections of retinal tissue were washed with PBS and then mounted. To visualize cell nuclei, counterstaining with 4',6-diamidino-2-phenylindole (DAPI) was carried out.

To assess the localization of labeled rhCNTF, immunofluorescence staining was carried out. First, slides with fixed sections of retinal tissue were washed with PBS. After subsequent incubation with blocking buffer, slides were incubated with primary polyclonal antibody against Iba-1 overnight at 4°C. Next day, the slides were repeatedly washed with PBS and incubated with Alexa Fluor™ 568-conjugated secondary antibody. Slides were then incubated in DAPI diluted in PBS to visualize cell nuclei. Finally, slides were washed repeatedly with PBS, dried out, embedded with antifade reagent, and closed with cover glasses.

To observe the penetration and assess the localization of labeled rhCNTF, mounted sections were imaged using a microscope equipped with a digital camera, and the acquired images from multiple explant areas subsequently manually inspected for the presence of fluorescence.

Sections from bovine retinal explants were incubated for 1 h at room temperature in blocking buffer, followed by overnight incubation at 4°C with rabbit antibody against Collagen IV to assess ILM integrity. Next, sections were stained with Alexa Fluor™ 488-labeled goat anti-rabbit secondary antibody, and to visualize cell nuclei, counterstained with Hoechst dye for 1 h at room temperature.

Sections were mounted with Vectashield® and prepared for imaging. As before, mounted sections were imaged using a microscope equipped with a digital camera, and the presence of fluorescence assessed manually from multiple explant areas to observe penetration of labeled rhCNTF.

4.4 Accelerated protein development (publication III)

To construct a novel production platform for streamlined pharmaceutical protein development and bioconjugation, we integrated cell-free protein synthesis (CFPS) with *Nostoc punctiforme* DnaE split intein-mediated capture, and UV-light triggered release.

4.4.1 Cell-free protein synthesis

Various CFPS systems were utilized for recombinant protein production. Whereas lysates prepared from different *E. coli* cell strains and wheat germ extract were also tested, for hCNTF-*Npu*DnaE$_{\Delta C16}$ production, CFPS systems utilizing tobacco plant bright yellow 2 cell lysates (BYL) (Buntru *et al.,* 2015) and mammalian HeLa cell lysates (1-Step Human Coupled IVT kit; Thermo Fisher Scientific, USA) were utilized. Whereas in certain developed cell-free systems the *in vitro* transcription and translation reactions are carried out separately, both CFPS systems used here are supplemented with an RNA polymerase, coupling the transcription of the template DNA to mRNA with the translation of the mRNA to protein in the same system. Overall, the reaction mixtures provide a supply of e.g., nucleotides, amino acids, and

energy substrates, as well as the accessory proteins and enzymes required in the energy metabolism, transcription, translation, and protein folding during recombinant protein synthesis.

CFPS systems were used according to published protocols and manufacturer's instructions. Briefly, CFPS of hCNTF-*Npu*DnaE$_{\Delta C16}$ was carried out by first mixing the required reaction components, followed by addition of plasmid DNA template. Prepared BYL CFPS reactions were incubated at 25°C shaken for up to 20 h in 96-well plates, whereas HeLa CFPS reactions were incubated stationary at 30°C for 6 h in microcentrifuge tubes. The quality of each CFPS reaction was checked by using enhanced green fluorescent protein (eGFP) as a positive control protein parallel to the CFPS reactions of the samples. Expression levels were also compared to a negative control where no template DNA was added to otherwise complete reaction mixtures. The synthesized target proteins in the complex CFPS matrix were used in subsequent experiments immediately without storage.

4.4.2 Photocleavage of peptides

To study the photocleavage of synthesized *Npu*DnaE$_{C16}$ peptides, i.e. C-inteins with various incorporated linkers and conjugates (**Table 5**), peptide samples were dissolved in ultra-pure water and reduced with tris(2-carboxyethyl)phosphine (TCEP) at 27°C for 30 min and then exposed to UV$_{365nm}$ light for 0–30 min.

Table 5. *Synthesized NpuDnaE$_{C16}$ peptides. Peptides with photoreactive groups marked in* ***bold****.*

	Peptide sequence	**Incorporated moieties**
PEP1	DGHNFALKNGFIASNCFGSKHis$_6$	N/A
PEP2	DGHNFALKNGFIASNCF-X-GSKHis$_6$	X = F(2-NO$_2$) = 2-Nitrophenylalanine
PEP3	DGHNFALKNGFIASNC-X-GSKHis$_6$	X = Fmoc-(S)-3-amino-3-(2-nitrophenyl)propionic acid
PEP4	DGHNFALKNGFIASNCF-X-GSKHis$_6$	X = Fmoc-photo-linker (CAS 162827-98-7)
PEP5	DGHNFALKNGFIASNCGSG-X-GGHis$_6$	X = Biotin on the side chain of lysine
PEP6	DGHNFALKNGFIASNCGSG-XY-GGHis$_6$	X = Biotin on the side chain of lysine Y = Photo-linker CAS 162827-98-7
PEP7	DGHNFALKNGFIASNCGSG-XY-GGEPEA	X = Biotin on the side chain of lysine Y = Photo-linker CAS 162827-98-7
PEP8	DGHNFALKNGFIASNCGSG-X-Y-GSKHis$_6$	X = Propargylglycine; Y = F(2-NO$_2$)

Generated peptide fragments were separated with an HPLC system and their elution monitored with UV$_{216nm}$ detection. The peptides were applied to a C18 (150 × 4.6 mm, 5 µm) column maintained at 25°C and a flow rate of 1.5 ml/min was used for all separations. Samples diluted in water were injected into the column using water (A) and acetonitrile (ACN; B), both with 1% trifluoroacetic acid, as eluents. The same gradient program (0–14 min 1% → 40% B, 14–16 min 40% → 70% B, 16–16.1 min 70% → 1% B, followed by 14 min equilibrium at 1% B) was used to elute all samples. Resulting chromatograms were examined and processed and a decrease in the area

under the curve (AUC) of the respective main peptide peak was used as a surrogate to measure light triggered cleavage of the peptides.

4.4.3 Capture and release

Having a pivotal role in the capture and release of expressed protein, the applicability of the intein-mediated protein *trans* splicing (PTS) of the artificially split *Npu*DnaE intein (*Npu*DnaE$_{\Delta C16}$ / *Npu*DnaE$_{C16}$) was studied.

To verify that the association and auto-excision of the folding intein domain can indeed ligate flanking exteins, in this study 1) a C-terminal peptide sequence containing e.g. a photocleavable moiety and/or a purification tag to 2) expressed protein of interest, the following experiments were set up. PTS reactions between photocleavable PEP2 / PEP3 (**Table 5**) and CFPS reactions containing expressed hCNTF-*Npu*DnaECΔ16 in the CFPS reaction matrix were carried out for 3 h at room temperature. The resulting putative C-terminally His$_6$-tagged hCNTF was captured with magnetic, Ni^{2+}-beads; handling of the magnetic beads was carried out with a magnetic purification system (KingFisher^{TM}, Thermo Fisher Scientific). Unbound proteins and CFPS reaction components were subsequently removed from the beads by repeated washing-steps. Photocleavage-mediated release of hCNTF was carried out with UV_{365nm} light for 0–6 h.

Utilizing the intein ligation reaction itself in the capture of the expressed protein was also evaluated. In this setup, PEP3 was first immobilized on the Ni^{2+}-beads. PTS reaction between immobilized PEP3 and CFPS expressed hCNTF-*Npu*DnaE$_{\Delta C16}$ was carried out overnight in room temperature to capture and immobilize the resulting ligation product, hCNTF-His$_6$, on the magnetic beads. As before, unbound proteins and CFPS reaction components were removed by repeated washing, and the release of hCNTF carried out by photocleavage with UV_{365nm} light for 0–6 h.

To verify that the capture and release were indeed achieved via the intein-mediated ligation and photocleavage, respectively, control experiments were carried out. A mock capture experiment was carried out using only the CFPS expressed hCNTF-*Npu*DnaE$_{\Delta C16}$ to rule out non-specific binding to the beads. After repeated washing to remove unbound proteins and components of the CFPS reaction, 'release' was carried out by photocleavage with UV_{365nm} light for 0–6 h. Similarly, to verify that the release takes place through cleavage of the photolabile groups, a mock release experiment was carried out. First, the non-photocleavable PEP1 was immobilized on the Ni^{2+}-beads, followed by PTS reaction between immobilized PEP1 and CFPS expressed hCNTF- *Npu*DnaE$_{\Delta C16}$ carried out overnight in room temperature, resulting in the immobilization of the ligation product, hCNTF-His$_6$, on the magnetic beads. After removal of unbound proteins and components of the CFPS reaction by wash steps, 'release' of hCNTF was carried out by photocleavage with UV_{365nm} light for 0–360 min.

As described in detail in publication **III,** detection of expressed, captured and released hCNTF was carried out with Western blotting (WB).

4.4.4 Bioconjugation

PTS reactions were carried out between CFPS expressed hCNTF-*Npu*DnaE$_{\Delta C16}$ and *Npu*DnaE$_{C16}$ PEP1, PEP5, PEP6 or PEP7 (**Table 5**). First, to assess if the respective modifications affect the PTS reaction, for example through steric hindrance, the biotinylated PEP5, PEP6 and PEP7 were used, whereas the non-modified PEP1 was used as a positive control for the PTS reaction. Briefly, HeLa CFPS reaction matrix containing expressed hCNTF-*Npu*DnaE$_{\Delta C16}$ was mixed with PEP1, PEP 5, PEP6 or PEP7 (all pre-reduced with TCEP) and the PTS allowed to take place in room temperature for 2 h. Reactions were halted with the addition of Laemmli sample buffer.

In a similar setup, to verify transferring biotin onto the protein of interest *via* PTS, the biotin-carrying PEP5 was used whilst PEP2 was used as a negative control. PTS reactions between these peptides and CFPS expressed hCNTF-*Npu*DnaE$_{\Delta C16}$ were set up as described above and carried out at room temperature overnight. Taking advantage of the strong, SDS-resistant binding between streptavidin and biotin, a streptavidin gel-shift assay (adapted from Fairhead and Howarth ADD and Sorensen) was used to visualize the transfer of biotin moiety to hCNTF. The described PTS reactions yielding the putative non-biotinylated and biotinylated hCNTF products were mixed with streptavidin, and the streptavidin-biotin binding then allowed to take place for 3 hours in room temperature. WB was carried out to detect protein expression and to determine the impact of biotinylation and streptavidin-binding on the electrophoretic migration of the target protein.

5 Study I: Codon optimization and factorial screening for enhanced soluble expression of human ciliary neurotrophic factor in Escherichia coli

6 Study II: Characterization and stability studies of recombinant human CNTF and its permeation in the neural retina in *ex vivo* organotypic retinal explant culture models

7 Study III: Accelerated pharmaceutical protein development with integrated cell free expression, purification, and bioconjugation

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8 Summary of the main results

The central findings of the publications are described in the following sections. Detailed results are found in the original publications.

8.1 Soluble expression of rhCNTF in *E. coli* (publication I)

To account for codon usage bias, a recognized obstacle with heterologous protein expression in foreign hosts, the hCNTF gene was codon optimized for expression in *E. coli*. To pinpoint suitable conditions for the soluble expression of rhCNTF, among *E. coli* bacterial cell strains and culture conditions, different fusion partners for enhanced protein solubility/folding were also included into the screening. PCR-amplified gene inserts and restriction enzyme-linearized plasmids were used to construct 9 different expression plasmids using Gibson Assembly™. After blue/white-screening of colonies, the incorporation of the hCNTF gene was confirmed via PCR using tag- and insert-specific primers. After propagation of the positive clones, the plasmid DNA was purified with Mini-preps and stored at -20°C for later use.

Screening of soluble expression was carried out in a total of 54 different combinations of culture conditions, culture media, bacterial cell strains, and fusion partners; rhCNTF overexpression was notably observed in all screened expression condition combinations.

To increase production of soluble rhCNTF for future experiments, expression was scaled up using pOPINF-hCNTF construct with autoinduction media, TBONEX at 25°C. After purification, 112 mg of soluble rhCNTF per ml culture medium was obtained, an 8- to 9-fold increase in soluble fraction and 10- to 20-fold increase in overall yield compared to earlier published reports. The function – and indirectly the correct folding – of purified rhCNTF was verified in an ELISA binding assay with the cognate receptor CNTFRα, with an estimated EC_{50} of 36 nM.

In summary, codon optimization combined with screening of expression conditions identified numerous conditions for producing soluble, functional rhCNTF. Generally, this approach is attractive for the high throughput expression screening of (pharmaceutically relevant) proteins.

8.2 rhCNTF characterization and stability (publication II)

Circular dichroism was utilized to study the structure of purified rhCNTF. As observed in the obtained spectra, a highly α-helical secondary structure and thus correct folding of rhCNTF was confirmed.

To ensure that the purified rhCNTF was not just able to bind to its cognate α receptor, but also biologically active and able to trigger downstream signaling, an *in vitro* cell-based study was carried out. Observed as BrdU incorporation, rhCNTF supported the short-term proliferation of the CNTFRα expressing cell-line TF-1.CN5a.1 with an estimated EC_{50} of 19 pg/ml (0.8 pM).

As previously used phosphate-buffered rhCNTF formulation showed a considerable loss of active protein upon freeze-thawing, buffer optimization was carried out using differential scanning fluorometry, also known as ThermoFluor, to screen for formulations enhancing rhCNTF stability in storage. Serving as a signal for

increased thermodynamic stability upon heating, elevated T_h was observed in several screened buffer, salt, and pH conditions, identifying potential storage conditions for rhCNTF. Out of the several potential formulations, clear fluorescence responses and high T_h estimates were observed with 100 mM sodium citrate, pH 5.6 and 100 mM MES, 500 mM NaCl, pH 7.0, reflecting high thermal stability. These formulations were chosen for follow-up studies.

Conformational stability of rhCNTF was retained in both buffers during storage on ice at +4°C and frozen at -80°C. Small amounts of high molecular weight species were detected in both storage conditions in the MES-based formulation, whereas only negligible amounts were found in the citrate formulation. No apparent loss of protein upon freeze-thawing was observed in either buffer.

In summary, we verified that expressed and purified rhCNTF was properly folded and biologically active. Screening identified buffer formulations enhancing rhCNTF stability, which can be used as a basis for further development through screening of e.g. additives to further improve the stability of rhCNTF.

8.3 Retinal permeation of labeled rhCNTF (publication II)

To investigate whether rhCNTF can permeate the retina to reach target cells, experiments with *ex vivo* organotypic retinal explants prepared from bovine and rat eyes war carried out to imitate *in vivo* ocular administration of the protein. 24 h after apical application – imitating intravitreal administration – labeled rhCNTF penetrated the inner limiting membrane and permeated further into the neural retina in organotypic *ex vivo* retinal explants prepared from both bovine and rat eyes. Studied only on explants from rat eyes, permeation into the retina after basolateral application – imitating periocular administration – was not observed. Seen as co-localization with Iba-1 immunopositivity, rhCNTF permeation to retinal microglia was observed. The labeled protein's permeation to retinal layers with CNTFRα expressing cells supports the possibility for direct neuroprotective actions. More generally, the *ex vivo* retinal explants have potential use in the systematic evaluation of the underlining determinants and factors of intravitreal biologicals' retinal permeation.

8.4 Accelerated protein development (publication III)

NEBuilder® was used to construct expression plasmids from PCR-amplified target protein genes and inverse PCR-amplified vector backbones for recombinant protein expression in BYL and HeLa-based CFPS. The insertion of target protein genes was first assessed with colony PCR, followed by verification with DNA sequencing. After propagation, plasmid DNA was purified with Midi-preps and stored at -20°C for later use.

The protein of interest, rhCNTF-*Npu*DnaE$_{C\Delta16}$, was expressed in both BY-2 and HeLa CFPS systems with yields of 20 mg/ml and 49 mg/ml, respectively. The protein *trans* splicing reaction was observed to yield C-terminally tagged target protein, verifying that upon association and folding, the cleavage of the auto-catalytic intein domain does take place in the complex CFPS matrices.

The efficiency and rate of light triggered photocleavage of the synthesized *Npu*DnaE$_{C16}$ peptides after UV_{365nm} irradiation was assessed using HPLC. The rate of

cleavage of less complex peptides was highest with PEP3 and PEP4, and lowest with PEP2, whereas with the bulkier peptides highest with PEP6 and PEP7, and lowest with PEP8 (**Table S3**). In general, rate of cleavage was slower with the bulkier peptides.

Whether utilizing the *Npu*DnaE$_{C16}$ peptide immobilized on a solid support or first in solution to generate the *de novo* affinity-tagged target protein, the PTS reaction with CFPS expressed rhCNTF-*Npu*DnaE$_{C\Delta16}$ yielded His$_6$-tagged rhCNTF captured on magnetic Ni^{2+}-beads. With the photocleavage-mediated release of rhCNTF to our chosen formulation, all included steps were completed in under 24 h, providing a proof-of-concept for our developed platform for streamlined protein production. Furthermore, moieties incorporated in the synthesized C-intein peptides were not observed to disrupt the PTS reaction and PTS successfully transferred biotin from PEP5 to rhCNTF as seen via binding to streptavidin.

In summary, a proof-of-concept was provided for our developed platform integrating split intein-mediated capture, light triggered release, and bioconjugation, shown as streamlined production of model protein hCNTF. The platform may have potential applications in the rapid screening of other pharmaceutically relevant proteins.

9 Discussion

After the first biological, Humulin®, was granted market approval in the early 1980's, hundreds of different biologicals have since been developed and used for treating different diseases. Biologicals are the fastest growing class of pharmaceuticals and are in widespread use in various fields of medicine today. In ophthalmology, anti-VEGF biologicals, such as ranibizumab, bevacizumab, and aflibercept, have become standard treatments for wet AMD and DME, diseases featuring pathological growth of abnormal blood vessels. Whereas anti-VEGF therapies are effective in most patients, in certain cases, drug response declines over time and some patients fail to respond altogether (Yang *et al.,* 2016). Furthermore, as these IVT injected biologicals have limited intraocular half-lives, their effects are only transient. Successful anti-VEGF therapy therefore commonly requires long-term follow-up, repeated administration, and commitment to the planned treatments. It is unsurprising that patient compliance with these treatments is sub-optimal. Thus, longer acting treatments circumventing or at least allowing for less frequent IVT dosing are in great demand and are actively pursued in biological drug development.

With the several anti-VEGF biologicals currently in development, new treatments for neovascular diseases will most likely emerge. Non-angiogenic posterior eye segment diseases, however, still suffer from a lack of effective treatments, especially such that would target disease pathogenesis. Moreover, as the number of AMD patients is projected to increase from 196 million to 288 million between 2020 and 2040 globally (Wong *et al.,* 2014), and as in the US the prevalence of retinal diseases is expected to more than double between 2010 and 2050 (**Fig. 8**), there is great urgency to develop new treatments for these diseases.

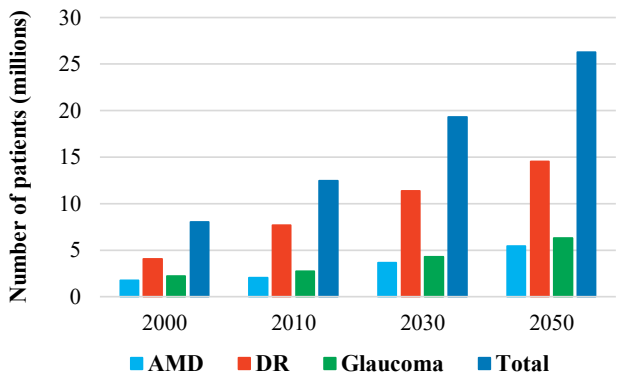

Figure 8. *Prevalence of most common retinal diseases causing vision loss and legal blindness in the US. Data and projections obtained from National Eye Institute website https://nei.nih.gov/learn-about-eye-health/resources-for-health-educators/eye-health-data-and-statistics.*

Ever since the pioneering work of Rita Levi-Montalcini and her peers it has been widely perceived that neurotrophic factors hold immense promise as potential therapies against neurodegenerative diseases. However, rapid enzymatic clearance and physiological barriers present formidable obstacles to delivering (neuroprotective) biologicals to targets in the CNS (Thorne & Frey, 2001). Neurodegenerative diseases

in the back of the eye, a region of the CNS more readily accessible for local drug delivery, represent attractive targets for neuroprotective factors (Pardue & Allen, 2018). Furthermore, as degeneration of different retinal neurons is a common feature of most posterior segment diseases regardless of disease etiology, neuroprotective therapeutic approaches are considered more universally applicable.

Much to the chagrin of patients and research community, various obstacles have hampered the progress to develop neuroprotective biologicals for ophthalmic use. Of the many proteins with recognized neurotrophic potential, none have yet translated from preclinical research to available treatments for posterior segment diseases nor any other neurodegenerative diseases. As abundant obstacles and open questions remain in the way of harnessing the potential of these and many other therapeutically potential proteins, there is ample room for innovation and improvements. This thesis focused on different protein production and development approaches while also touching on the pharmacokinetics, and in particular the retinal permeation of ophthalmic biologicals using human CNTF as an ophthalmologically relevant model protein. In the following sections, the used methods and obtained results and their implications will be discussed.

9.1 Therapeutic protein production and development

Production. The production of high protein quantities can be a significant roadblock, especially during the early stages of biological drug research and development. Considerable efforts are put into addressing the involved obstacles by developing and improving methods and techniques for protein expression and purification. However, as each protein can be considered unique, oftentimes also in their production, therapeutic protein production workflows are designed and developed in part as dictated by the protein. In other words, although drug developers and researchers have a wide and growing palette of different tools at their disposal, in order to meet the requirements of individual proteins, considerable process customization and optimization is commonly necessary, and often determined empirically (Berkmen, 2012).

Recombinant protein production can be carried out in different expression hosts ranging from simple bacteria and yeast to considerably more sophisticated insect, plant, and mammalian systems (Singha *et al.,* 2017). Even though the available systems differ vastly, for example, in their costs, complexity, speed, and achievable yields, the choice is not only dependent on these 'external' factors. For example, overexpression of heterologous recombinant proteins in the simplest system, *E. coli,* often takes place in a microenvironment alien to the target protein (Rosano & Ceccarelli, 2014). This can also overwhelm the host's intracellular protein folding and proteostasis machinery, leading to the expressed protein accumulating as insoluble inclusion bodies (Singha *et al.,* 2017). Complicated refolding is necessary for purifying soluble recombinant protein from inclusion bodies, and as this may still yield protein in a misfolded and aggregation prone state (Gąciarz *et al.,* 2017; Sarker *et al.,* 2019), potentially leading to immunogenicity (Thatcher, 1990), avoiding inclusion body formation is highly desirable. Moreover, *E. coli* is limited in its capacity to form PTM's such as disulfide bonds correctly (Berkmen, 2012; Gąciarz *et al.,* 2017) and it is not capable of performing glycosylation on proteins (Peti & Page, 2007). As such,

features can be critical for many properties of proteins, such as proper folding, activity, stability, and pharmacokinetics, the requirements for the expression host are critically important and dictated by the properties of an individual protein. For example, glycosylated mAbs require expression in PTM-competent mammalian hosts, and as they comprise the majority of biologicals in development, mammalian-based systems (e.g. CHO cells) have become prominently used (Walsh, 2018).

Regardless of its shortcomings, using *E. coli* for recombinant protein expression is desirable owing to its notable advantages. As it has been utilized for decades, the use of *E. coli* is well established with plenty of available resources and tools. Furthermore, *E. coli* can be rapidly cultured, easily handled and its genetic manipulation is well-established. Since it is also cost-effective, *E. coli* is often a first choice as a protein expression host (Berkmen, 2012; Rosano & Ceccarelli, 2014; Jia & Jeon, 2016). *E. coli* is currently utilized in the production of around 30% of biologicals on the market (Singha *et al.,* 2017).

As the correct parameters for protein expression are difficult to predict, producing high protein yields is often a trial and error process (Berkmen, 2012). Therefore, in setting up a production pipeline for a protein of interest, small-scale testing of several expression hosts, expression constructs, and culture conditions has become a commonly adopted strategy to aid in selecting the most favorable conditions. As rapid progress is made in this field, reviews discussing 'try-first' strategies and approaches for enhancing recombinant protein production have been compiled (Peti & Page, 2007; Gräslund *et al.,* 2008; Rosano & Ceccarelli, 2014; Jia & Jeon, 2016; Singha *et al.,* 2017).

The manufacture of cytokine therapeutics can be challenging (Lipiäinen *et al.,* 2015). Although these proteins have been studied since the late 1970's, many efforts to produce the neuropoietic cytokine hCNTF in *E. coli* resulted in low yields and/or required protein purification and refolding from insoluble inclusion bodies (Krüttgen *et al.,* 1995; McDonald *et al.,* 1995; Di Marco *et al.,* 1996; Narhi *et al.,* 1997; Wagener *et al.,* 2014). Here, by utilizing codon optimization and screening of different factors, such as culture media, solubility enhancing fusion tags, and bacterial strains, we found suitable conditions for the expression of soluble rhCNTF in *E. coli*. These conditions were later utilized in production scale-up to produce high amounts of functional rhCNTF (publication **I**). Whereas the functional activity of purified rhCNTF was first indirectly verified through binding with the cognate alpha receptor (publication **I**), the correct folding and biological activity were later observed with circular dichroism and in a cell proliferation assay, respectively (publication **II**).

Stability. The environmental conditions that biologicals experience during production, i.e. synthesis in living cells, purification and formulation, are very different compared to their native environments. During these processes, biologicals are exposed to both chemical and physical stresses that can affect the conformational stability of the proteins; compared to the native milieu and conditions the changes in the protein environment can be considerably more dramatic during production. These stresses may perturb the native state, ultimately resulting in partial or complete unfolding of the protein structure. As protein functionality depends on its conformation and stability, the structural denaturation can lead to diminished or lost activity (Radhakrishnan *et al.,* 2017). Stresses that can induce therapeutic protein

degradation and denaturation include physical factors (e.g. pressure, shear stresses, temperature) and chemical changes (e.g., oxidation, deamidation, disulfide modifications, fragmentation) (Manning *et al.,* 2010; Jiskoot *et al.,* 2012; Radhakrishnan *et al.,* 2017). Formation of protein aggregates is another common outcome from structural unfolding. The aggregation results from *de novo* interactions between newly exposed protein regions.

Aside from its production challenges, *in vitro* CNTF is not stable at room and body temperature (Hottinger & Aebischer, 1999; Fandl *et al.,* 2006). For example, decreased solubility and increased propensity to aggregate upon unfolding have been observed with hCNTF (Narhi *et al.,* 1997). Furthermore, covalent dimer formation via unpaired cysteine residue has been proposed to lead to hCNTF aggregates and precipitation (Fandl *et al.,* 2006). More generally, the manufacture of cytokine therapeutics is often challenging due to their propensity to aggregate and adhere to surfaces, which is postulated to arise from their hydrophobicity (Lipiäinen *et al.,* 2015), an attribute of cytokines' helical bundle fold (Ricci & Brems, 2004). It is reasonable to assume that such attributes also play a role in the stability of the similarly folded hCNTF. To this end, modifications to CNTF's structure have been made to generate variants with improved therapeutic activity, but also improved solubility and stability (Panayotatos *et al.,* 1993; Fandl *et al.,* 2006). In our study, phosphate-based storage formulation was not suitable for purified rhCNTF, resulting in significant loss of protein upon freeze-thawing. Therefore, we had to find a more suitable liquid formulation.

A wide variety of analytical methods can be utilized to characterize proteins and to assess their stability (den Engelsman *et al.,* 2011; Jeong *et al.,* 2012; Filipe *et al.,* 2013). Here, a spectrum corresponding to a highly helical protein was first observed with circular dichroism (CD), confirming the correct folding of produced rhCNTF (publication **II**). Whereas low-throughput methods such as CD and differential scanning calorimetry can be used to assess proteins' conformational stability, we wanted to do this with rhCNTF's in numerous formulations, and therefore opted to use differential scanning fluorimetry as it is amenable for higher throughput (Filipe *et al.,* 2013). This method, more commonly known as ThermoFluor, is a thermal shift assay that measures thermal unfolding of proteins using different fluorescent dyes as reporters (Kopec & Schneider, 2011; Sviben *et al.,* 2018). Commonly used fluorescent dyes, such as SYPRO Orange® and bis-ANS, bind to hydrophobic residues and regions in proteins. In an aqueous environment their fluorescence is quenched, but upon increasing environment hydrophobicity (e.g. during thermal protein unfolding), the dyes are consequently able to fluoresce upon interacting with exposed hydrophobic regions, leading to dramatic increases in the fluorescence signal (Pantoliano *et al.,* 2001; Boivin *et al.,* 2013). The temperature of hydrophobic exposure (T_h) can be derived from the obtained fluorescence signal in this indirect measurement of unfolding; T_h correlates well with the melting temperature, T_m, observed with orthogonal methods (Seabrook & Newman, 2013). ThermoFluor has other applications in drug discovery (Pantoliano *et al.,* 2001), optimization of protein purification, characterization (Boivin *et al.,* 2013), crystallization (Sviben *et al.,* 2018), protein stabilization (Seabrook & Newman, 2013), and in assessing the formation and stabilization of protein-protein complexes (Kopec & Shneider, 2011). Moreover, using alternative dyes further broadens the method's applicability: thiol-

specific dyes that react with accessible cysteines upon unfolding and dyes for monitoring of protein aggregate formation have been developed (Boivin *et al.,* 2013).

Here, we utilized SYPRO Orange® in a ThermoFluor screen with various buffer, salt, and pH conditions to determine changes in the T_h and conformational stability of rhCNTF (publication **II**). Based on the screen, two buffers with high T_h estimates were chosen for rhCNTF storage. The protein's storage stability was subsequently monitored with ThermoFluor and dynamic light scattering (DLS); whereas ThermoFluor was used to monitor for changes in the T_h, DLS was utilized to detect higher molecular weight species, such as protein aggregates. In DLS, the rate of scattering fluctuations over time are measured; as these scattering rate fluctuations relate directly to diffusivities and hydrodynamic size, DLS can be used to assess the presence of particles of different sizes, such as protein aggregates, in liquid formulations (Philo, 2009). However, DLS is not quantitative, cannot distinguish between species of similar sizes, and is fairly inaccurate with non-spherical species. Therefore, it is most suited for rapid screening for the presence of aggregates (Philo, 2009; den Engelsman *et al.,* 2011; Filipe *et al.,* 2013). In general, it is recommended that different orthogonal methods, such as analytical ultracentrifugation and size-exclusion chromatography are utilized for more detailed characterization of aggregates in protein formulations.

Based on observed thermal stability and negligible amounts of formed aggregates, the citrate buffer appears to be the optimal formulation for further development of rhCNTF (publication **II**). As the presence of even trace protein aggregates can be critical risk factors for patient safety (Jiskoot *et al.,* 2012), a more thorough analysis and characterization of aggregation is warranted, and further development should also focus on elimination of any aggregation in the CNTF formulation(s). Additionally, albeit based on preliminary observations rhCNTF remains stable in isolated vitreous at 37°C for 44 h (data not shown), post-administration aggregation *in oculo* cannot be ruled out without further studies.

Cell-free expression. Producing therapeutic proteins with cell-free protein synthesis (CFPS) is compelling as these systems offer numerous advantages over traditional cell-based expression systems (Casteleijn *et al.,* 2013; Khambhati *et al.,* 2019). For example, with the need for cell lysis removed, CFPS systems are rapid, open and more flexible, and they can facilitate the expression of toxic and difficult-to-express proteins while also allowing the incorporation of unnatural amino acids that can be utilized in bioconjugation and protein engineering. On the other hand, there are notable challenges that limit further use of the systems. As lysate preparation disrupts the cellular machinery that is needed for proper glycosylation patterns, CFPS systems are limited in their ability to perform this PTM. Despite considerable cost reductions achieved over the decades, CFPS is still costly compared to alternative protein expression systems (Casteleijn *et al.,* 2013; Gao *et al.,* 2019; Khambhati *et al.,* 2019); for example, expressing rhCNTF yields comparable to those achieved in *E. coli* (publication **I**) with the commercial HeLa-cell based system would have required a budget in excess of 1 million euros. Based on recent marketing approvals the biopharmaceutical industry's interest in CFPS is still to be invoked; instead of exploring, developing, and adopting new systems, tried-and-true expression hosts are still favored (Walsh, 2018). Although producing therapeutic proteins at industrial

scales may not yet be universally feasible with CFPS (Gao *et al.,* 2019), as many of the recognized issues and shortcomings are being addressed, these systems are evolving rapidly (Castleijn *et al.,* 2013; Khambhati *et al.,* 2019). As such, they are far from reaching their full potential, and they can be expected to become alternatives and even replacements to cell-based systems for the production of biologicals in the future. Here, the expression of ophthalmologically relevant neurotrophic proteins CNTF (publication **III**) and PEDF (data not shown) was achieved using CFPS.

Whereas state-of-the-art protein production in cells typically involves numerous downstream and upstream process steps and can take from weeks to months, here we described a potential integrated platform for streamlined production/screening of biologicals. We provided a proof-of-concept for our solution integrating CFPS with intein-mediated capture and UV-light release using rhCNTF as a model protein (publication **III**; **Fig. 9**). Moreover, transfer of biotin to the target protein was achieved with our system.

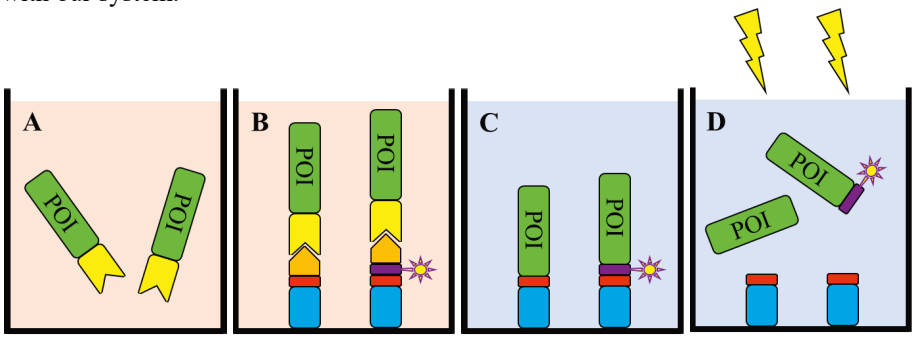

Figure 9. *Diagram of the integrated protein production workflow. (A) Cell-free protein synthesis of the protein of interest (POI) fused with a C-terminal NpuDnaE$_{CΔ16}$ N-intein (yellow); (B) Protein trans splicing (PTS)-mediated capture of POI. The synthesized NpuDnaE$_{C16}$ C-intein capture peptides (orange-(purple)-red-blue) are either in solution or immobilized to a surface (latter scenario depicted). The red, purple, and blue segments represent a photo-cleavable amino acid/linker, an unnatural amino acid linked to a reactive moiety, and an affinity tag, respectively. At this stage the split intein will autocatalytically splice itself out upon folding; (C) After PTS, the cell-free expression matrix and side products are removed and the final buffer/formulation added; (D) UV-light triggered release of POI. Bioconjugation with the reactive moiety may be carried out with immobilized POI after stage C, with released POI after stage D, or even later.*

At this stage our aim was to condense and streamline protein production times to a fraction of the time compared to traditional approaches, as described, for example, in our production workflow with *E. coli* cells (publication **I**). Therefore, we did not assess the folding or activity of the purified rhCNTF, nor did we study whether the release with high energy UV light had any detrimental effects on the protein. As such, the described work was the first step of a planned platform with integrated analytical instruments and modules for measuring and monitoring, e.g., the amount, activity, integrity, and immunogenicity of expressed proteins. Additionally, as the platform can be used for the directed bioconjugation of proteins, the concept might be expanded by utilizing alternative inteins, peptides, and incorporated moieties to open further possibilities for protein engineering. For example, better control over glycosylation, PEGylation, and conjugation of other probes could be achieved as the number and location of target sites for these modifications could be more precisely engineered with the system.

9.2 Retinal penetration of biologicals

Certain aspects in the ocular pharmacokinetics of biologicals are still poorly established. Whereas several recent studies have elucidated how different molecular attributes influence the vitreous half-life of biologicals (Shatz *et al.,* 2016; Crowell *et al.,* 2019), the picture on retinal permeation and posterior elimination of biologicals is less clear. Studies done with different biologicals and other macromolecules have given rise to varying and at times contradicting propositions for the determinants and limiting factors of retinal permeation.

Whereas molecular weight (Mr) was initially proposed as the major determinant (Mordenti *et al.,* 1999), this has later been disputed as permeation of the full retinal thickness has been observed with several biologicals larger than the proposed exclusion limits (El Sanharawi *et al.,* 2010). Retinal permeation is considered to depend on the retinal anatomy and physiology as well as on the molecular attributes of individual biologicals (e.g. molecular weight, charge, hydrophobicity, and hydrodynamic size) (El Sanharawi *et al.,* 2010; Balaratnasingam *et al.,* 2015). Interestingly, as unequal retinal penetration has been observed with biologicals of similar sizes (Shahar *et al.,* 2006; Heiduschka *et al.,* 2007), it is likely that retinal penetration does not depend only on size nor mere passive diffusion. Both binding specificities and active transport mechanisms have been postulated to influence retinal permeation of protein drugs (El Sanharawi *et al.,* 2010). The influence of the proposed factors have, however, mostly been speculated, and any systematic quantitation of their contributions is yet to be carried out. As such, research on the retinal penetration of biologicals has predominantly been carried out with qualitative methods (e.g. immunohistochemistry, microautoradiography) and there are still sparse quantitative estimates of the retinal penetration of biologicals (del Amo *et al.,* 2017). Moreover, retinal permeation studies with biologicals have most often been done with animal eyes and, therefore, translational value to the diseased human eyes remains uncertain (Eng & Kertes, 2006).

Ophthalmic biologicals with smaller molecular weights and hydrodynamic sizes have been developed, since they can be formulated at higher molar doses. It is also assumed that smaller biologicals would permeate into the retina more efficiently. However, it is unclear how deep biologicals need to penetrate into the retina to achieve therapeutic activity. Since, for example, anti-VEGF biologicals may elicit their action by binding extracellular VEGF outside the retina (del Amo *et al.,* 2017), it is not certain whether retinal penetration is in fact a requirement with all modalities. Clearly, regardless of their increasing use in treating ocular conditions, the retinal permeation of biologicals is poorly studied and inadequately understood. There are substantial gaps in our understanding of the underlying factors and their significance in retinal penetration.

To our knowledge, no reports on the retinal penetration of the small protein CNTF (nor its engineered analogue Axokine®) have been published to date. Here, we utilized organotypic *ex vivo* retinal explant cultures to study retinal permeation with purified, labeled rhCNTF (publication **II**). We showed that rhCNTF readily permeates across the ILM and distributes into the neural retina. Even though ILM has been suggested to be an obstacle for intravitreal CNTF therapy (Müller *et al.,* 2009), it did not block retinal permeation of rhCNTF.

The main benefit of using such organotypic cultures is that in comparison to e.g. *in vitro* cell cultures and primary cell cultures, the intricate structure and cell connections of the retina are retained and the explants better reflect the *in vivo* situation (Valdés *et al.,* 2016; Alarautalahti *et al.,* 2019). Since there are anatomic differences between the eyes of different species (Peynshaert *et al.,* 2017), retinal explants prepared from both rat and bovine eyes were used in this study for comparison. However, as the structure of the retina has been observed to change upon aging and degeneration, retinal permeation in such situations may be altered and should ideally be studied with models that better reflect this. Considerable differences depending on pathologies, disease stage and severity may, however, make disease effects challenging to study. Nonetheless, on one hand a possible limitation of our study is that the experiments were done with retinas obtained from healthy animals and the results may therefore not reflect permeation in pathological conditions nor in aged retinas. On the other hand, PK studies in healthy animals are considered valid as evidence points to disease effects in fact being insignificant, (del Amo *et al.,* 2017), and studies utilizing explants from healthy eyes should therefore be valid.

In a similar fashion with recent studies evaluating the influence of different factors on the intravitreal pharmacokinetics of biologicals (Shatz *et al.,* 2016; Crowell *et al.,* 2019), we see that the retinal explants have potential to be utilized in systematically evaluating the determinants of retinal permeation and further in developing biologicals capable of overcoming the various barriers therein. Based on preliminary observations in rat retinal explants, we have also seen penetration past the ILM into the neural retina with a set of other small model proteins (data not shown).

9.3 Retinal effects and delivery of CNTF

Retinal effects. Supportive and neurotrophic actions of CNTF against retinal damage and neurodegeneration have been demonstrated in several studies with different animal disease models (MacDonald *et al.,* 2007; Wen *et al.,* 2012). Although the protein has been widely studied for decades now, the exact molecular mechanisms of its neuroprotective actions on retinal neurons are still not fully understood. Whereas observed to elicit neuroprotection on different retinal neurons *in vitro*, this is considered to mostly occur indirectly, by modulating retinal glial cells to a more neuroprotective phenotype and inducing them to express and release other neuroprotective factors (Wen *et al.,* 2012).

Perhaps the most accepted theory is the so-called Müller hypothesis, in which the effects of CNTF in the retina are proposed to be mediated by inducing Müller glia – cells that envelop retinal neurons and provide them metabolic and homeostatic support – to express and secrete cytokines that elicit neuroprotection on photoreceptors and other cells (Rhee *et al.,* 2013). CNTF treatment leads to activation of JAK-STAT and MAPK cascades in Müller cells both *in vitro* (Sarthy *et al.,* 2016) and *in vivo* (Peterson *et al.,* 2000), and induces extensive transcriptional changes *in vivo*, involving cytokines and growth factors (Xue *et al.,* 2011). Interestingly, induction of both pro- and anti-inflammatory agents was detected in flow-sorted Müller cells after IVT administration of CNTF (Xue *et al.,* 2011). Such widespread response may account for the complex effects that CNTF elicits on retinal neurons. Furthermore, the Müller hypothesis has gained additional support as continuous CNTF

treatment demonstrated efficacy in a phase II trial in patients with MacTel 2, a condition associated with Müller cell abnormalities and dysfunction (Wubben *et al.,* 2018).

Apart from observed effects on Müller glia, CNTF may also modulate retinal astrocytes to adopt a more neuroprotective phenotype (van Adel *et al.,* 2005). Furthermore, *in vitro,* the effects of exogenous CNTF on RGCs were observed to be mediated partially via the induction of endogenous CNTF expression in astrocytes (Müller *et al.,* 2009), which have also been identified to be a major source of factors mediating the beneficial effects of inflammation in the eye (Leibinger *et al.,* 2009).

Although incompletely studied, it is possible that CNTF has similar effects on the third retinal glial cell type, microglia. These resident macrophages of the retina are commonly regarded to possess dual roles, being able to elicit both detrimental and protective effects on neurons (Wang *et al.,* 2011; Rathnasamy *et al.,* 2019). On one hand, they can support cell survival and repair by secreting growth factors, and on the other hand they can participate in inflammation by secreting pro-inflammatory cytokines, such as TNF-α and various interleukins, as seen in the retina. Although microglia-mediated inflammation is increasingly linked to retinal neurodegeneration, there is also evidence supporting a neuroprotective role of microglia (Karlstetter *et al.,* 2015; Rashid *et al.,* 2019). Therefore, how retinal microglia affect neurons and their survival may strongly depend on context.

Microglia are increasingly thought to play a central part in the pathophysiology of several retinal diseases (Karlstetter *et al.,* 2015; Rashid *et al.,* 2019), and activation and accumulation of retinal microglia have been observed, for example, in DR (Cunha-Vaz *et al.,* 2014), AMD and RP (Glybina *et al.,* 2010). Additionally, microglial activation and migration to the outer retina and subretinal space has been observed with age, possibly in response to local inflammation and tissue insults in the retina (Chen *et al.,* 2010). Thus, modulation of microglia, and the glia-glia network by-and-large, has been studied as a therapeutic strategy for mitigating retinal neurodegeneration and inflammation (Rashid *et al.,* 2019; Rathnasamy *et al.,* 2019). For instance, sustained delivery of a low-dose corticosteroid markedly inhibits microglial responses (Glybina *et al.,* 2010). As microglia have dual roles, modulating their action for neuroprotection is a complex task, requiring careful balancing; whereas microglial suppression may prevent the detrimental effects, simultaneous suppression of their neuroprotective action is unwanted and should be carefully evaluated in any therapy with microglial targets (Harada *et al.,* 2002; Rashid *et al.,* 2019)

The direct role of CNTF on microglia and its effects on the crosstalk with Müller cells and the glia-glia and glia-neuron interactions (Harada *et al.,* 2002; Wang *et al.,* 2011) are still unclear. Nonetheless, although to the best of our knowledge neither CNTFRα expression nor responses to CNTF have been studied in human retinal microglia, both are plausible. Direct effects on retinal microglia are supported here as labelled rhCNTF permeated into the retina and co-localized with microglia in the rat retinal explants (publication **II**), and as microglial responses to exogenous rat CNTF treatment have been observed in rat eyes (Cen *et al.,* 2007). Furthermore, in terms of morphology and function, retinal microglia are comparable to their counterparts in the brain (Rathnasamy *et al.,* 2019) and CNTF has been shown to influence CNS microglia in rodents, with CNTFRα expression observed in cultured primary microglia isolated from rat (Krady *et al.,* 2008) and murine CNS (Lin *et al.,* 2009),

and in human CNS microglia (Baek *et al.,* 2018). Rat CNS microglia expressed and secreted trophic factors in response to CNTF (Krady *et al.,* 2008), whereas in murine CNS microglia the response was weakly pro-inflammatory (Lin *et al.,* 2009). It is not certain whether postulated pro-inflammatory responses elicited by exogenous CNTF in the human retina would be detrimental since CNTF has also been implied to mediate beneficial effects in the retina (Fischer, 2008). CNTF's actions on retinal microglia in both healthy and diseased tissue should be investigated in order to gain insight into the mechanisms behind CNTF's neuroprotective actions on retinal neurons. Research should be performed with human retinal tissue if possible.

Apart from retinal glia, CNTF may elicit retinal protective effects via actions on the RPE (Li *et al.,* 2011). The RPE effects were shown only *in vitro* in primary cultures of human fetal RPE cells and proof of *in vivo* effects is still missing. It is possible that CNTF's effects on the RPE do not need to be direct but may instead be mediated, e.g., by retinal glia that in response to exogenous CNTF express and secret other factors promoting the survival of RPE. Nonetheless, even though we did not observe permeation of labeled rhCNTF across the whole explant (publication **II**), permeating to the RPE in > 24 h to elicit direct effects cannot be ruled out without further studies.

As most retinal diseases involve activation of Müller glia and reactive gliosis (Xue *et al.,* 2011), CNTF may be considered a rather universal treatment approach for retinal neurodegeneration, even though its efficacy in earlier clinical trials in GA and RP patients was insufficient. Due to the often complex etiologies of retinal diseases, their successful treatment may require multiple therapeutics with different modes of action. In this context, complex actions of CNTF on retinal glia are of utmost interest. Further, as seen with CNTF and GDNF treatment with axotomized RGCs *in vivo* (Flachsbarth *et al.,* 2018), it is realistic to assume that activating multiple trophic signaling pathways with the co-administration of CNTF and other growth factors could result in synergistic treatment effects.

In summary, effects of CNTF on retinal glia and neurons are complex and are incompletely understood. Neuroprotective effects of CNTF on retinal neurons may have both direct and indirect glial cell-mediated components. Permeation of labeled rhCNTF into the neural retina shows that the protein distributes to layers with recognized CNTF-responsive cells (publication **II**), implying that the protein's availability to such cells is not a limitation to any direct actions.

Retinal delivery of CNTF. As biologicals fail to permeate to the posterior eye segment after topical administration and since the blood-ocular barriers prevent their entry from the circulation, direct intravitreal administration is necessary (Shatz *et al.,* 2018). As there are risks involved with IVT administration and as many posterior segment diseases require repeated administration, there is an urgent and unmet need to develop methods to prolong the effects and injection intervals for biologicals.

With CNTF, various approaches for sustained intraocular delivery have been explored (Ghasemi *et al.,* 2018). For example, an approach where CNTF expressed as a fusion protein with a binding partner was injected together with a hydrogel that was covalently modified with a corresponding binding target, resulting in a sustained intravitreal release of bioactive CNTF over at least one week (Delplace *et al.,* 2019). Furthermore, an engineered fusion protein consisting of hCNTF and sCNTFRα, termed Hyper-CNTF, was shown to act as a super-agonist on gp130 and LIFRβ

expressing cells, and it was speculated that as it was lacking a synthetic peptide linker it would not be recognized as non-self nor be immunogenic (März *et al.,* 2002). ‘as this fusion protein can affect non-CNTFRα expressing cells, it would be of great interest to study whether it elicits more universal trophic effects on retinal cells than CNTF alone. Since Hyper-CNTF has a M_r of over 80 kDa, its intravitreal half-life is expected to be longer compared to CNTF, although this may also have an impact on the retinal permeation of the protein.

Renexus® is the approach closest to reaching the clinical practice. Initially showing only moderate efficacy in GA and RP patients (Zhang *et al.,* 2011; Kauper *et al.,* 2012), a recent phase II trial showed retinal degeneration, photoreceptor loss and reading speed loss were significantly reduced in Renexus® treated eyes in patients with MacTel 2 (Chew *et al.,* 2019). Such differences in drug efficacy against different retinal conditions may be explained by their etiologies. Unlike other conditions, MacTel 2 is associated with Müller cell pathologies and thus better coincides with the proposed actions of CNTF (Wubben *et al.,* 2018).

Although Renexus® has proceeded to phase II studies in glaucoma patients with phase III studies in MacTel 2 patients pending, the implantation and implant removal requires precise surgery. There is still need for less invasive approaches for the sustained intraocular delivery of CNTF and other biologicals. Furthermore, in addition to the various stresses imparted on biologicals during production processes, they may be exposed to various harsh and destabilizing stresses also during the preparation of drug delivery systems as well as during intravitreal drug release (Jiskoot *et al.,* 2012). Therefore, it is necessary to carry out characterization of the protein and its stability as well as extensive scrutinization for the potential for immunogenicity and aggregate formation in the context of the developed delivery systems.

9.4 General considerations for the future

There is a pressing need for developing new treatments for the neurodegenerative diseases of the retina, as most of them still lack efficient treatments. As these diseases often exhibit a very slow and (in earlier stages) asymptomatic progress, developed treatment strategies should match such disease progression. Furthermore, patient stratification is of paramount importance, although the clinical assessment of the symptoms and disease status is not always straightforward in the heterogenous and complex retinal diseases. Therefore, one of the central challenges is to define the optimal timeframe for treatments; on one hand to pinpoint when treatments should be initiated to achieve maximal benefits, and on the other hand when disease progression is too advanced for clinically meaningful outcomes with therapeutic intervention. Hence, the identification and monitoring of clinically relevant endpoints and biomarkers is essential to benchmark and monitor disease progression and efficacy of therapies.

There are currently numerous ophthalmologic biologicals in development. As their retinal penetration remains poorly understood, elucidation of the involved factors could greatly benefit the research in both academia and industry. Herein, we envision the *ex vivo* retinal explants utilized to systematically investigate how different molecular attributes and pathological changes affect the retinal permeation of different

biologicals. Increased understanding of the retinal permeation could be utilized in the rational design and engineering of new biologicals for ophthalmology.

Regardless of remarkable advances in the field, the development and production of recombinant proteins is often time-consuming and frequently requires process optimization to achieve desirable yields of a protein with adequate stability and functionality. Furthermore, features like immunogenicity cannot be properly studied during preclinical animal studies and, thus, they are often revealed only later during clinical trials. Therefore, it is important to develop methods for early detection of immunogenicity of lead biologicals. Then, different proteins and engineered variants could be tested early on to alter and abolish immunogenicity.

Protein aggregates are major contributors to immunogenicity of therapeutic proteins. Although this phenomenon is widely recognized, it is incompletely defined and complex. Aggregation takes place via different simultaneous mechanisms at varying rates but is also highly dependent on the protein in question. As various stress factors during protein production and storage can induce the formation of the aggregates, early identification of aggregation propensity in candidate molecules is of interest. To achieve this, it is necessary to define relevant descriptors for protein stability and aggregation that can be monitored, for example, with different spectroscopic techniques during production. Different approaches could be utilized during early development to rapidly screen for the druggability and producibility of candidate biologicals in a smaller scale, and later to help avoid aggregate formation during larger scale processing.

All in all, early detection of immunogenic and aggregation propensity in therapeutic proteins in the initial phases of development is essential in addressing and mitigating their impacts. As both are common culprits for clinical trial failures, an altogether better understanding of these phenomena could help in thwarting their impact, direct the development process, and curb the costs therein. In conclusion, combining various established methodologies with new and innovative approaches is likely required to break Eroom’s law and to keep the development of new pharmaceuticals sustainable.

10 Conclusions

This thesis focused on the development of biologicals and on their use in ophthalmic conditions, using rhCNTF as a model protein. The following key conclusions were reached:

1. Upon codon optimization and factorial screening, various suitable conditions for the soluble expression of rhCNTF in *E. coli* were found.

2. An optimized approach was chosen for the larger scale expression of rhCNTF. High yields of soluble, correctly folded, and biologically active rhCNTF were purified.

3. Upon screening of suitable formulations for the long-term storage of rhCNTF, two were chosen for further studies. Stability assessment with rhCNTF indicates citrate buffer to be the best candidate for further development.

4. Labeled rhCNTF permeates into the neural retina in both explant models in 24 h. Direct neuroprotective effects on CNTFRα expressing RGCs and photoreceptors are therefore possible.

5. rhCNTF is expressed in both CFPS systems, with a proof-of-concept for intein-mediated capture and light-mediated release of rhCNTF demonstrated.

6. Our protein production platform may be utilized for the streamlined bioconjugation of proteins, as shown with the biotinylation of CFPS expressed rhCNTF.

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Appendix

Table S1. *Non-exhaustive summary of novel biologicals in development for retinal diseases. Updated in January 2020.*

	Biological	Structure	Target	Modality	Posology	Indication	Status	Outcomes	Ref.
Clinical stage	**Brolucizumab (Beovu® RTH258 ESBA1008)**	ScFv fragment	VEGF-A	Anti-angiogenic	IVT	wet AMD, DME	Phase III in wet AMD patients completed (NCT02307682, NCT02434328); phase III studies in DME patients recruiting (NCT03481634, NCT03917472)	Noninferior to aflibercept with prolonged duration of effect in wet AMD patients; FDA approval in October 2019 for treating wet AMD with EMA approval pending	Radhakrishnan *et al.,* 2017; Dugel *et al.,* 2019; Iyer *et al.,* 2019; Markham, 2019
	Abicipar pegol (AGN-150998)	DARPin®	VEGF-A	Anti-angiogenic	IVT	wet AMD, DME	Phase III in wet AMD patients completed (NCT02462486, NCT02462928); phase II in DME patients completed (NCT02186119)	Prolonged effect but also higher rates of adverse events compared to ranibizumab, EMA assessment for wet AMD therapy pending; phase III trials in DME patients planned	Callanan *et al.*, 2018; Al-Khersan *et al.,* 2019
	OPT-302	Fc-fusion protein	VEGF-C and VEGF-D	Anti-angiogenic	IVT	wet AMD, DME	Phase II in wet AMD patients completed (NCT03345082); phase Ib/IIa in DME patients ongoing (NCT03397264)	Meaningful visual gains in earlier phase I/IIa study; pivotal phase III studies expected	Mandal *et al.,* 2018; Opthea Ltd., 2018; Al-Khersan *et al.,* 2019; Opthea Ltd., 2019
	Faricimab (RG7716 RO6867461)	Humanized bispecific mAb	VEGF-A and ANG-2	Anti-angiogenic	IVT	wet AMD, DME	Phase III ongoing in wet AMD (NCT03823287, NCT03823300); DME patients (NCT03622580, NCT03622593)	Superior clinical improvements compared to ranibizumab in earlier phase II trials; phase III results pending	Chakravarthy *et al.,* 2017; Kaplon & Reichert 2018; Al-Khersan *et al.,* 2019
	Nesvacumab (REGN910)	Human mAb	ANG-2	Anti-angiogenic	IVT	wet AMD, DME	Phase II completed in wet AMD (NCT02713204) and DME patients (NCT02712008)	No significant improvements as a co-therapy with aflibercept compared to aflibercept; discontinued	Wang & Lo 2018; Al-Khersan *et al.,* 2019
	Sonepcizumab (iSONEP™)	Humanized mAb	Sphingosine-1-phosphate	Anti-angiogenic	IVT	wet AMD	Phase II completed (NCT01414153)	Inadequate efficacy as monotherapy and in combination with anti-VEGF therapy; discontinued	Sabbadini, 2010; Lpath Inc., 2015; Volz & Pauly 2015
	Carotuximab (DE-122)	Chimeric mAb	Endoglin	Anti-angiogenic	IVT	wet AMD	Phase II ongoing (NCT03211234)	Results of co-administration with ranibizumab pending	Kaplon & Reichert 2018; Tracon Pharma, 2018
	Rinucumab (REGN2176)	Human mAb	PDGFRβ	Anti-angiogenic	IVT	wet AMD	Phase II completed (NCT02418754)	Co-administration with aflibercept showed no additional efficacy over aflibercept monotherapy	Dunn *et al.,* 2017; Al-Khersan *et al.,* 2019; Iyer *et al.,* 2019
	Teprotumumab (RG1507)	Human mAb	IGF-1R	Anti-angiogenic	IV	DME, thyroid eye disease	Phase I completed in DME patients (NCT02103283)	Results unpublished	Agarwal *et al.,* 2015; Stewart, 2017; Kaplon & Reichert 2018

Table S1 continued.

	Biological	*Structure*	*Target*	*Modality*	*Posology*	*Indication*	*Status*	*Outcomes*	*Ref.*
Clinical stage	**Volociximab (M200)**	Chimeric mAb	$\alpha_5\beta_1$ integrins	Anti-angiogenic	IVT	wet AMD	Phase I (NCT00782093)	Results of co-administration with ranibizumab sparcely published; no further studies underway	Ishikawa *et al.,* 2015; Volz & Pauly 2015; Iyer *et al*., 2019
	Daclizumab (Zenapax®)	Humanized mAb	IL-2Rα	Immuno-modulation	IV	wet AMD	Phase II completed (NCT00304954)	Less anti-VEGF IVT injections required; ocular use has not been explored further	Nussenblatt *et al.,* 2010; Volz & Pauly 2015
	Tocilizumab	Humanized mAb	IL-6R	Immuno-modulation	IV	DME	Phase II withdrawn (NCT02511067)	Trial with ranibizumab vs. tocilizumab and tocilizumab co-therapy with ranibizumab never initiated	Agarwal *et al.,* 2015; Stewart, 2017
	Lampalizumab	Humanized Fab-fragment	Complement factor D	Complement inhibition	IVT	GA secondary to AMD	Phase III completed (NCT02247479, NCT02247531)	Ineffective as a treatment; discontinued	Volz & Pauly 2015; Holz *et al.,* 2018; Kassa *et al.,* 2019
	Eculizumab (Soliris®)	Humanized mAb	Complement factor C5	Complement inhibition	IV	GA secondary to AMD	Phase II completed (NCT00935883)	No effect on disease progression; no further studies initiated	Volz & Pauly 2015; Iyer *et al.,* 2019; Kassa *et al.,* 2019
	Tesidolumab (LFG-316)	Human mAb	Complement factor C5	Complement inhibition	IV, IVT	GA secondary to AMD, wet AMD	Phase II completed (NCT01535950, NCT01527500)	No effect on improving vision nor reducing lesion growth; discontinued	Volz & Pauly 2015; Kassa *et al.,* 2019
	ICON-1 (hI-CON1)	Antibody-like Fc immunoprotein	Tissue factor	Anti-angiogenic, anti-inflammatory	IVT	wet AMD	Phase II completed (NCT01485588, NCT02358889) and ongoing (NCT03452527)	Visual gains comparable to ranibizumab with fewer required treatments observed in earlier phase II studies; results of co-therapy with and maintenance therapy after aflibercept pending	Hu 2018, Wells *et al.,* 2018; Iyer *et al.,* 2019
	PEDF	Serpin	PEDFR	Anti-angiogenic, Neuroprotection	Local gene transfer, IVT	wet AMD, Glaucoma, RP	Phase I completed with retinal gene transfer (NCT00109499)	Intravitreal PEDF decreased photoreceptor cell death and protects RGCs; gene transfer inhibits neovascularization	Tombran-Tink & Barnstable 2003b; Campochiaro *et al.,* 2006; Comitato *et al.,* 2018; Pardue & Allen 2018; Devoldere *et al.,* 2019
	Renexus® (NT-501 CNTF)	Cytokine-secreting encapsulated cells	CNTFRα, gp130, LIFRβ	Neuroprotection	Intravitreal implant	GA, RP, Glaucoma, MacTel 2	Phase II study ongoing in glaucoma patients (NCT02862938); phase III studies in MacTel 2 patients enrolling (NCT03316300, NCT03319849)	No obvious clinical improvements in GA and RP patients; disease progression retarded in MacTel 2 patients	Kauper *et al.,* 2011; Birch *et al.,* 2016; Chew *et al.,* 2019

Table S1 continued.

	Biological	*Structure*	*Target*	*Modality*	*Posology*	*Indication*	*Status*	*Outcomes*	*Ref.*
Clinical stage	**hNGF (Cenegermin, Oxervate®)**	Neurotrophin	TrkA, $p75^{NTR}$	Neuroprotection	IVT, Topical	wet AMD, Glaucoma, RP	Phase I in glaucoma patients completed (NCT02855450); phase II in RP patients completed (NCT02609165)	Subjective improvements in glaucoma and RP patients treated with NGF eye drops reportedd	El Sanharawi *et al.,* 2010; Kimura *et al.,* 2016; Rocco *et al.,* 2018
	GSK933776	Humanized mAb	Amyloid β	Reduction of fibrils in drusen	IV	GA secondary to AMD	Phase II completed (NCT01342926)	No clinically meaningful improvements; did not slow lesion growth	Volz & Pauly 2015; Rosenfeld *et al.,* 2018
Preclinical stage	**Affibodies®**	Small proteins	VEGFR-2	Anti-angiogenic	*In vitro*	wet AMD		VEGF-A signaling blocked	Fleetwood *et al.,* 2014; Fleetwood *et al.,* 2016
	Tanibirumab	Human mAb	VEGFR-2	Anti-angiogenic	IVT	wet AMD		Choroidal neovascularization partially suppressed	Kim *et al.,* 2014
	Ramucirumab (Cyramza ®)	Human mAb	VEGFR-2	Anti-angiogenic	N/A	wet AMD		Proposed; no published studies	Falcon *et al.,* 2016
	Nanobody® BI-X	Trispecific sdAb	VEGF, ANG-2, and albumin	Anti-angiogenic	IVT	wet AMD		Prolongation of vitreal half-life	Fuchs & Igney 2017
	BDNF	Neurotrophin	TrkB, $p75^{NTR}$	Neuroprotection	IVT	Glaucoma, RP		Protective effects on RGCs, RPE, and photoreceptors	Afarid *et al.,* 2016; Kimura *et al.,* 2016; Pietrucha-Dutczak *et al.,* 2018
	GDNF	Neurotrophic factor	GFRα1, Ret	Neuroprotection	Local gene trasnfer, IVT	Glaucoma, RP		Protection of RGCs and photoreceptors (indirectly?)	Ejstrup *et al.,* 2012; Afarid *et al.,* 2016; Kimura *et al.,* 2016; Pietrucha-Dutczak *et al.,* 2018
	MANF	Neurotrophic factor	Not known	Neuroprotection	IVT	Glaucoma, RP		Protection of RGCs and photoreceptors	Gao *et al.,* 2017; Lu *et al.,* 2018
	HSPs	Chaperone	Protein folding & misfolding	Oxidative stress defence	*In vitro,* IVT	AMD, Glaucoma		Protective effects on RGCs, RPE, and photoreceptors	Yu *et al.,* 2001; Urbak & Vorum 2010; Pietrucha-Dutczak *et al.,* 2018

AMD, age-related macular degeneration; ANG, angiopoietin; BDNF, brain-derived neurotrophic factor; CNTF, ciliary neurotrophic factor; CNTFR, CNTF receptor; DARPin, designed ankyrin repeat protein; DME, diabetic macular edema; Fab, antigen-binding fragment; Fc, fragment, crystallizable; GA, geographic atrophy; GDNF, glial-cell-line derived neurotrophic factor; GFR, GDNF family receptor; HSP, heat shock protein; IGF-1R, insulin-like growth factor receptor 1; IL, interleukin; IV, intravenous; IVT, intravitreal; LIFR, leukemia inhibitory factor receptor; mAb, monoclonal antibody; MacTel, macular telangiectasia; MANF, mesenchephalic astrocyte-derived neurotrophic factor; NGF, nerve growth factor; p75NTR, low-affinity neurotrophin receptor; PDGFR, platelet-derived growth factor receptor; PEDF, pigment epithelium-derived factor; PEDFR, PEDF receptor; RGC, retinal ganglion cell; RP, retinitis pigmentosa; RPE, retinal pigment epithelium; ScFv, single-chain antibody fragment; sdAb, single-domain antibody; Trk, tropomyosin receptor kinase; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor

Table S2. *Examples of ocular drug delivery approaches in development for biologicals.*

	Drug delivery system	*Materials/ structure*	*Biologicals*	*Posology*	*Features & outcomes*	*Status*	*Ref.*
Carriers	**Microspheres**	Biodegradable & non-biodegradable polymers (EVA, PEG, PLA, PLGA, PVA)	Bevacizumab, GDNF, ranibizumab	IVT	Bevacizumab release from micro- and nanospheres over 90 days; harsh fabrication conditions complicate retaining protein integrity and activity during and after manufacture; initial burst release	Preclinical (GDNF); several phase I trials initiated with anti-VEGF-loaded particles	El-Sanharawi *et al.,* 2010; Li *et al.,* 2012; García-Caballero *et al.,* 2017; Radhakrishnan *et al.,* 2017; Iyer *et al.,* 2019
	Nanospheres		Aflibercept, bevacizumab, CNTF, bFGF, ranibizumab	IVT, periocular			Nkansah *et al.,* 2008; El-Sanharawi *et al.,* 2010; Li *et al.,* 2012; Joseph & Venkatraman 2016; Radhakrishnan et al., 2017; Kelly *et al.,* 2018; Lau *et al.,* 2018
	Hydrogels	Alginate, chitosan, dextran, HA, PEG	Aflibercept, bevacizumab	IVT, suprachoroidal	Hydrogels often well tolerated in eyes of study animals; therapeutic concentrations of biologicals maintained for extended periods	Cross-linked PEG formulation for sustained aflibercept delivery in preclinical development	Delplace *et al.*, 2015; Yu *et al.*, 2015; Lau *et al*., 2018
	Composite systems	Particles within particles/hydrogel	Aflibercept, bevacizumab, ranibizumab	IVT	Particles within particles/gels; sustained release of biologicals over months	Preclinical	Agrahari *et al.,* 2016; Joseph & Venkatraman 2016
	Liposomes	Phospholipids, cholesterol, PEG	Bevacizumab, ranibizumab	IVT, periocular	Sustained ocular residency; retaining protein integrity and activity challenging during drug encapsulation	Preclinical	Delplace *et al.,* 2015; Joseph & Venkatraman 2016; Agrahari *et al.,* 2017; Joseph *et al.,* 2017; Radhakrishnan et al., 2017

Table S2 continued.

	Drug delivery system	*Materials/ structure*	*Biologicals*	*Posology*	*Features & outcomes*	*Status*	*Ref.*
Implants	**Encapsulated cell technology**	Semipermeable, nonbiodegradable membrane surrounding polymer-encapsulated, genetically engineered cells that express and secrete therapeutic proteins	NT-501: CNTF, NT-503: soluble VEGFR-Fc fusion protein	Surgical implantation	Sustained production and intravitreal delivery of biologicals over years	NT-501 progressing into phase III trials in MacTel 2 patients; NT-503 discontinued due to high incidence of patients requiring intravitreal anti-VEGF injections	Al-Khersan *et al.,* 2019; Chew *et al.,* 2019
	Port Delivery System	Semipermeable, nonbiodegradable and refillable reservoir implant with a transscleral port	Ranibizumab	Trans-scleral implantation, refill via port	Comparable efficacy to frequent IVT ranibizumab injections with sustained ranibizumab release for up to a year	Phase III studies in wet AMD patients recruiting (NCT03677934, NCT03683251)	Al-Khersan *et al.,* 2019; Campochiaro *et al.,* 2019;
	Posterior MicroPump Drug Delivery system	Microelectromechanical pump system with a trans-conjunctival port	Ranibizumab	Subconjunctival implantation, refill via port	Drug delivery in nanodroplets; difficulties with implantation, challenges with the precise amount of intravitreally released drug	Studied in a prospective small-scale clinical trial	Humayun *et al*., 2014; Lau *et al.,* 2018

AMD, age-related macular degeneration; CNTF, ciliary neurotrophic factor; EVA, ethylene vinyl acetate; bFGF, basic fibroblast growth factor 2; GDNF, glial cell-line derived neurotrophic factor; HA, hyaluronic acid; IVT, intravitreal; MacTel, macular telangiectasia; PEG, polyethylene glycol; PLA, polylactic acid; PLGA, polylactic-co-glycolic acid; PVA, poly(vinyl alcohol); VEGFR, vascular endothelial growth factor receptor

Table S3. *Photocleavage of NpuDnaE$_{C16}$ peptides.*

Peptide	Cleavage time (min)	% of main peak AUC remaining	t_r of main peak (min)
PEP2	0	100	10.75
	5	72	10.75
	15	58	10.75
	30	39	10.77
PEP3	0	100	10.61
	5	26	10.62
	15	16	10.61
	30	8	10.60
PEP4	0	100	11.83
	5	22	11.84
	15	11	11.84
	30	9	11.84
PEP6	0	100	11.15
	5	41	11.07
	15	40	11.08
	30	30	11.06
PEP7	0	100	11.87
	5	43	11.88
	15	39	11.78
	30	25	11.78
PEP8	0	100	10.61
	5	55	10.62
	15	42	10.62
	30	22	10.62